ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

EFFECT OF SILANIZATION AGENT ON THE IMMOBILIZATION OF LIPASE ON A SILICA-BASED SUPPORT MATERIAL

M.Sc. THESIS

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SİLİKA BAZLI TAŞIYICIYA LİPAZ İMMOBİLİZASYONUNDA SİLANLAMA AJANININ ETKİSİ

YÜKSEK LİSANS TEZİ

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To my family,

FOREWORD

I would like to present my gratitude to my thesis advisor Prof. Dr. Yüksel Güvenilir for her trust in me and her guidance throughout my study. I also want to thank to my laboratory colleagues Msc. Chem. Eng. Cansu Ülker and Chem. Eng. Ece Ulu for their intellectual input to this study and their help during my laboratory work. I am also very pleased to have the assistance of undergraduate students Işık Sena Akgün and İrem Zerey, in laboratory.

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ABBREVIATIONS

3-APTES	**:** 3-aminopropyl triethoxysilane
3-APTMS	**:** 3-aminopropyl trimethoxysilane
3-GPTMS	**:** 3-glycidyloxypropyl trimethoxysilane
Asp	**:** Aspartic acid
BET	**:** Brunauer-Emmett-Teller
CALB	**:** *Candida antarctica* lipase B
CALB L	**:** Free form of *Candida antarctica* lipase B
FT-IR	**:** Fourier transform infrared spectroscopy
His	**:** Histidine
PCL	**:** Poly(ε-caprolactone)
RHA	**:** Rice husk ash
ROP	**:** Ring opening polymerization
SEM	**:** Scanning electron microscopy
Ser	**:** Serine
TGA	**:** Thermal gravimetric analysis
U	**:** Enzyme activity unit
UV	**:** Ultroviolet
v/v	**:** Volume per volume ratio

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SUMMARY

Enzymatic syntheses for industrial applications have attracted attention recently due to their advantages over conventional chemical syntheses. Utilization of enzymes provides clean and environmental friendly processes in addition to high selectivity and specificity. However, in order to be adapted for industrial applications, enzyme should be engineered through several processes. Immobilization of enzyme is one of these engineering process and has become an attractive technique to improve enzyme stability and activity.

Immobilization is a technique which is applied in order to improve enzyme activity and stability. In general, interactions between the enzyme and a solid support material are desired so that the enzyme is localized in a specific region. The catalytic activity is conserved, even improved. Additionally, immobilization also permits repeated use of enzymes by recovery. This allows continuous and economical processes for industrial applications. It also prevents protein contamination of the product. There are several methods of immobilization, mainly physical adsorption, entrapment, covalent attachment and cross-linking. Physical adsorption is the simplest immobilization technique. It depends on weak interactions between the enzyme and a solid support material. Though it is an economic method, enzyme leakages due to weak interactions may cause difficulties. Entrapment is achieved by occlusion of the enzyme in a polymer matrix. The enzyme is protected from the effects of the environment. However, mass transfer limitations occur. The difference of this method is that the enzyme is not bounded to the polymer matrix. During covalent attachment, functional groups of the enzyme create strong covalent bonds. It is important to ensure that catalytic sites of the enzyme are not involved in binding. Cross-linking is achieved by the intermolecular linkages between the proteins or between the proteins and the solid support material.

A solid support material is required for the immobilization procedure on which the enzyme will be bound. A suitable support material should be considering several criteria. It should have resistance to physical forces, inertness towards enzymes. It should also be accessed easily and cheap, biocompatible and resistant to microbial contamination. Support material can be divided into two groups: organic and inorganic support materials. Support materials can be used after surface modification for improved results.

One of the most commonly used surface modification technique is silanization. In case of materials having hydroxylated surfaces, modification occurs through these hydroxyl groups and generally organosilanes are used as silanization agent. Hydroxyl groups on the surface react with active groups of the silanization agent, generally alkoxy groups. Trifunctional silanes have higher reactivity and monolayers of trifunctional silanes are believed to yield well-ordered and compact films. Most

widely used trifunctional organosilanes are 3-aminopropyl triethoxysilane (3-APTES), 3-aminopropyl trimethoxysilane (3-APTMS) and 3-glycidyloxypropyl trimethoxysilane (3-GPTMS).

Rice husk is high silica containing material which is obtained from rice production process as by-product. Silica is considered to be a suitable support material for enzyme immobilization. Additionally, it is a cheap and easily accessible material since it is a by-product. Utilization of this by-product may be considered as a waste management strategy.

Lipases may find various applications in different industries. For example they can be used as additives in detergents and responsible for removal of oil stains since they catalyze hydrolysis of fats. In pulp and paper industry, lipases are used for removal of pitch whose deposition reduces the quality of pulp. Lipases are also used to replace the conventionally used emulsifiers in food industry and for flavor development. Lipases can be mainly used for biodiesel production in energy industry in order to replace fossil fuels with renewable energy sources. Biopolymers are generally preferred for medical application since they are biodegradable and biocompatible with human body thus nontoxic. Poly (ε-caprolactone) (PCL) is a biodegradable and biocompatible polymer which has been extensively studied for medical applications. PCL is synthesized by ring opening polymerization (ROP) reaction of ε-caprolactone and the reaction can be catalyzed by lipase eznyme.

In this study, free *Candida antarctica* lipase B (CALB L) was immobilized on modified rice husk ash (RHA). By this procedure, it is aimed to increase catalytic activity and improve stability of CALB. RHA was chosen since it is an easily accessible and cheap material. Additionally, its high silica content makes RHA a suitable choice since it is known that silica is successfully used as a support material for enzyme immobilization.

As a first step, RHA was obtained by burning rice husks at 600 °C for 6 h. Surface modification was achieved by using two different silanization agents, 3-glycidyloxypropyl trimethoxysilane (3-GPTMS) and 3-aminopropyl trimethoxysilane (3-APTMS). The modified RHA were used as support materials for immobilization of CALB by physical adsorption. Enzyme loading ratio and concentration of silanization agent were evaluated as part of the optimization studies. Furthermore, pH, thermal, storage and operational stabilities were studied; optimum pH and temperature of the immobilized lipase samples were determined. Characterization of RHA, surface modified RHA and immobilized lipase samples were done by Fourier transform infrared spectroscopy (FT-IR), thermal gravimetric analysis (TGA), scanning electron microscopy (SEM) and Brunauer-Emmett-Teller (BET) surface area analysis.

FT-IR and TGA results showed that both 3-GPTMS and 3-APTMS modifications were successfully achieved. Surface area analysis also showed the textural structures of the support material and modified support materials. Immobilization of lipase on these modified samples was also successful; reaching immobilization efficiencies of 88.5 % and 90.8 % for 3-GPTMS and 3-APTMS, respectively. Catalytic activity of CALB was retained and specific activities were increased by approximately 1.8 folds for both types of immobilized enzyme samples. This value is close to the specific activity of a commercially available immobilized CALB, Novozyme 435® (2.2 times higher than free CALB). Stabilization experiments showed that the immobilize CALB samples have remarkable pH and temperature stability, and storage and

operational stabilities. Based on these results, it can be suggested that CALB immobilized onto 3-GPTMS and 3-APTMS silanized RHA may become an attractive alternative to other commercially available CALB.

Catalyzing polymerization reactions, especially lactone monomers, is one of the applications of immobilized lipases that attracted attention. Polycaprolactone (PCL) synthesis via enzymatic ring opening polymerization (ROP) reaction has been widely studied in recent years. There is a commercially available immobilized CALB, Novozyme 435®, which is known to be efficiently utilized for PCL polymerization. In further studies, these immobilized CALB onto 3-GPTMS and 3-APTMS silanized RHA may be adapted for PCL polymerization. These immobilized lipases may also be used in co-polymerization reactions with other monomers which may allow faster kinetics and yield polymer chains with higher molar masses. Such an application exists in literature.

SİLİKA BAZLI TAŞIYICIYA LİPAZ İMMOBİLİZASYONUNDA SİLANLAMA AJANININ ETKİSİ

ÖZET

Endüstriyel uygulamalarda enzimatik sentez reaksiyonları, geleneksel kimyasal sentezlerle karşılaştırıldığında sahip olduğu avantajlar nedeniyle son yıllarda ilgi çekmiştir. Enzimlerin kullanımı, yüksek selektivite ve spesifisiteye ek olarak temiz ve çevre dostu prosesleri mümkün kılar. Ancak, enzimlerin endüstriyel uygulamalara adapte edilebilmesi için çeşitli işlemlerden geçmesi gerekmektedir. Enzimlerin immobilizasyonu bu mühendislik işlemlerinden biridir ve enzim stabilitesini ve aktivitesini iyileştirmek için cazip bir teknik olmuştur.

İmmobilizasyon enzim aktivitesini ve stabilitesini iyileştirmek için uygulanan bir yöntemdir. Genel olarak, enzimin belirli bir bölgede sınırlandırılması için enzim ile katı destek materyali arasında kurulan etkileşimler istenir. Katalitik aktivite korunmasının yanı sıra artırılır. Buna ek olarak, immobilizasyon enzimin geri kazanma yoluyla tekrar tekrar kullanılmasına olanak sağlar. Bu durum, endüstriyel uygulamalar için sürekli ve ekonomik prosesleri mümkün kılar. Ayrıca, enzimin reaksiyon ortamından daha kolay uzaklaştırılmasını sağladığı için ürünün protein kontaminasyonuna da engel olur. Başlıca fiziksel adsorpsiyon, tutuklama, kovalent bağlama ve çapraz bağlama olmak üzere farklı immobilizasyon yöntemleri bulunmaktadır. Fiziksel adsorpsiyon en basit immobilizasyon yöntemidir. Enzim ile katı destek materyali arasında kurulan zayıf etkileşimlere dayanır. Ekonomik bir yöntem olmasına karşın, zayıf etkileşimlere bağlı olarak yaşanabilecek enzim sızıntısı sorunlara yol açabilir. Tutuklama enzimin bir polimer matriksine kapatılması ile gerçekleştirilir. Enzim bulunduğu ortamın neden olabileceği etkilerden korunur. Ancak, kütle transferi önünde engeller oluşur. Bu yöntemin diğer yöntemlerden farkı, enzimin polimer matriksine bağlanmıyor oluşudur. Kovalent bağlanma sırasında ise, enzimin fonksiyonel grupları güçlü kovalent bağlar oluşturur. Ancak, katalitik bölgelerin bu bağlanmalara katılmadığından emin olunması önemlidir. Çapraz bağlanma proteinlerin kendi arasında ya da proteinler ve katı destek materyali arasında kurulan moleküller arası bağlarla sağlanır.

İmmobilizasyon işlemi için enzimin bağlanacağı bir katı destek materyali gereklidir. Uygun bir destek materyalinin bazı kriterler göz önünde bulundurularak seçilmesi gerekir. Fiziksel güçlere karşı dayanıklı olmalı, enzimle reaksiyona girmemelidir. Ayrıca ucuz ve kolay elde edilebilir bir malzeme olmalı, biyouyumlu ve mikrobiyel kontaminasyona karşı dirençli olmalıdır. Destek materyalleri iki gruba ayrılabilir: organik ve inorganik destek materyalleri. Destek materyalleri daha iyi sonuçlar elde etmek adına yüzey modifikasyonu uygulandıktan sonra kullanılabilir.

En sık kullanılan yüzey modifikasyonu yöntemlerinden biri silanlamadır. Yüzeylerinde hidroksil grupları bulunan materyaller söz konusu olduğunda, yüzey modifikasyonu bu hidroksil grupları aracılığıyla gerçekleşir. Genellikle, organosilanlar silanlama ajanı olarak kullanılır.

Pirinç kabuğu, pirinç üretim prosesinin yan ürünü olup yüksek silika içeriğine sahip bir materyaldir. Silika enzim immobilizasyonu için uygun bir destek materyali olarak kabul edilmektedir. Ayrıca, hem ucuz hem de yaygın bir prosesin yan ürünü olduğu için kolay ulaşılabilen bir materyaldir. Bu materyalden yararlanılması bir atık yönetimi stratejisi olarak da görülebilir.

Lipaz enzimi farklı endütrilerde değişik uygulama alanları bulmaktadır. Örneğin, yağların hidroliz tepkimesini katalizledikleri için lipazlarlar deterjanlarda katkı maddesi olarak kullanılır ve yağ lekelerinin temizlenmesinden sorumludurlar. Kağıt endütrisinde ise lipazlar, biriktiğinde kağıt kalitesini düşüren zift maddelerinin arıtılmasında kullanılır. Ayrıca, gıda endüstrisinde, hem halihazırda kullanılan emülgatörler yerine ve tatlandırıcı olarak kullanlırlar. Enerji endüstrisinde, lipazlar fosil yakıtları yenilenebilir enerji kaynakları ile değiştirmek için başlıca biodizel üretiminde kullanılır. Lipaz enzimi biyouyumlu bir polimer olan polikaprolaktonun sentez tepkimesi olan halka açılması polimerizasyonunu katalizler. Polikaproklakton biyouyumlu olmasında dolayı özellikle medikal uygulamalar için oldukça sık çalışılan bir malzemedir.

Bu çalışmada, serbest *Candida antarctica* lipaz B (CALB L) yüzey modifikasyonu yapılmış pirinç kabuğu külü üzerine immobilize edilmiştir. Bu işlem ile lipaz enziminin katalitik aktivitesi artırılmak ve stabilitesi iyileştirilmek istenmiştir. Pirinç kabuğu külü ucuz ve kolayca temin edilebilen bir materyal olduğu için destek materyali olarak seçilmiştir. Ayrıca, içerdiği yüksek silika miktarı pirinç kabuğu külünü uygun bir seçim yapar çünkü silikanın enzim immobilizasyonunda destek materyali olarak başarılı bir şekilde kullanıldığı bilinmektedir.

İlk adım olarak, pirinç kabuğu külü, pirinç kabuklarının 6 saat boyunca 600 °C' de yakılması ile elde edildi. Yüzey modifikasyonu iki farklı silanlama ajanı kullanılarak gerçekleştirildi, 3-glisidiloksipropil trimetoksisilan (3-GPTMS) ve 3-aminopropil trimetoksisilan (3-APTMS). Fiziksel adsorpsiyon yöntemi ile CALB immobilizasyonu için destek materyali olarak yüzey modifikasyonu yapılmış pirinç kabuğu külleri kullanılmıştır. Optimizasyon çalışmalırının bir parçası olarak enzim yükleme oranının ve silanlama ajanı konsantrasyonunun immobilizasyon üzerine etkisi incelendi. Ayrıca, enzim örneklerinin pH ve sıcaklık stabiliteleri test edilmiş, raf ömrü, tekrarlı kullanım stabilitesi ve optimum pH ve sıcaklık değerleri belirlenmiştir. Pirinç kabuğu külünün, yüzey modifkasyonu yapılmış pirinç kabuğu külü örneklerinin ve immobilize edilmiş lipaz örneklerinin karakterizasyonu Fourier transform kızılötesi spektroskopisi (FT-IR), termal gravimetrik analiz (TGA), taramalı elektron mikroskobu (SEM) ve Brunauer-Emmett-Teller (BET) yüzey alanı analizi kullanılarak yapıldı.

FT-IR ve TGA sonuçları, 3-GPTMS ve 3-APTMS kullanılarak yapılan yüzey modifikasyonunun başarılı olduğunu göstermiştir. Lipazın bu taşıyıcılara immobilizasyonu da başarılı olmuştur; immobilizasyon verimleri sırasıyla 3-GPTMS ve 3-APTMS için % 88.5 ve % 90.8' e ulaşmıştır. Katalitik aktivite korunmuş ve spesifik aktivite değerleri her iki tip immobilize enzim örneği için yaklaşık 1.8 kat artırılmıştır. Bu değer, ticari olarak mevcut immobilize CALB'nin (Novozyme 435®) spesifik aktivite değerine yakındır (serbest CALB'nin 2.2 katı). Stabilizasyon deneyleri, immobilize olmuş CALB örneklerinin dikkate değer pH ve termal stabiliteleri, raf ömürleri ve tekrarlı kullanım stabiliteleri olduğunu göstermiştir. Bu sonuçlara dayanarak, 3-GPTMS ve 3-APTMS ile yüzey modifikasyonu yapılmış

pirinç kabuğu külü üzerine immobilize edilen lipaz enziminin ticari olarak mevcut bulunan immobilize lipazlara alternatif oluşturabileceği önerilebilir.

İmmobilize lipazların ilgi çeken uygulamalarından biri lakton polimerizasyonu başta olmak üzere polimerizasyon reaksiyonlarının katalizlenmesidir. Son yıllarda enzimatik halka açılması reaksiyonu ile polikaprolakton (PKL) sentezi geniş ölçüde çalışılmıştır. PKL sentezinde etkili bir şekilde kullanıldığı bilinen ticari bir immobilize lipaz enzimin bulunmaktadır (Novozyme 435®). Daha ileri çalışmalarda, 3-GPTMS ve 3-APTMS ile silanlamış pirinç kabuğu külü üzerine immobilize edilen bu lipaz örnekleri PKL polimerizasyon reaksiyonlarında kullanımı için adapte edilebilir. Bu lipazlar ayrıca lakton monomerlerinin yanı sıra diğer monomer çeşitleri ile beraber kopolimerizasyon reaksiyonlarında kullanılabilir. Bu, hızlandırılmış kinetiğe ve yüksek molar ağırlıklı polimer zincirlerinin sentezlenmesine olanak sağlayabilir. Böyle bir çalışma literatürde bulunmaktadır.

1. INTRODUCTION

Biocatalysis has long been seen as a promising area for chemical synthesis and its advantages over conventional chemical syntheses have long been recognized. However, industrial applications have been widely neglected. Technological developments and environmental concerns have provided a wide range of application areas for biocatalysis in industrial synthesis. Biocatalysis has many attractive aspects such as being biodegradable, tracking a reaction route that yields less waste than conventional chemical syntheses and which does not require activation or protection of any functional groups. Thus, it is both environmental friendly and economic process [1].

Enzymes have many advantageous features for production of wide variety of products under mild conditions. They also provide high chemo-, regio- and stereo-selectivities, specificity and activity. Enzymatic synthesis may find industrial applications in many fields including fine chemistry production and food and pharmaceutical industry [2-5]. However, enzymes are not very convenient for industrial applications due to some properties. They are soluble in water, they are not well functioning under non-physiological conditions and may be inhibited by reaction products [6]. However, the disadvantages can be eliminated through engineering.

Immobilization of enzymes is a technique to improve enzyme activity and stability. Enzyme immobilization is achieved by confining the enzyme molecules to a phase in which the substrates and the products are present and can be in contact. As well as improving activity and stability, immobilization also permits repeated use of enzymes by recovery. This allows continuous and economical processes for industrial applications [1, 2]. It also prevents protein contamination of the product [1, 4]. There are several methods of immobilization, mainly adsorption, entrapment, covalent attachment and cross-linking [7, 8]. Each method has its own advantages and disadvantages.

Immobilization procedure requires a support material on which enzyme will be bound. A suitable support material should be physically resistant, unreactive with enzymes, easily accessible and cheap, biocompatible and resistant to microbial contamination [3]. There are many organic and inorganic materials that are used as support material such as natural and synthetic polymers, minerals, zeolites, ceramics, silica and activated carbon [3, 9]. Support materials can be used after surface modification for improved results. Hydroxyl groups on the surface react with active groups of the silanization agent, generally alkoxy groups. Trifunctional silanes have higher reactivity and monolayers of trifunctional silanes are believed to yield well-ordered and compact films. Most widely used trifunctional organosilanes are 3-aminopropyl triethoxysilane (3-APTES), 3-aminopropyl trimethoxysilane (3-APTMS) and 3-glycidyloxypropyl trimethoxysilane (3-GPTMS).

Lipases may find various applications in different industries. For example they can be used as additives in detergents and responsible for removal of oil stains since they catalyze hydrolysis of fats. In pulp and paper industry, lipases are used for removal of pitch whose deposition reduces the quality of pulp. Lipases are also used to replace the conventionally used emulsifiers in food industry and for flavor development. Lipases can be mainly used for biodiesel production in energy industry in order to replace fossil fuels with renewable energy sources. Biopolymers are generally preferred for medical application since they are biodegradable and biocompatible with human body thus nontoxic. Poly (ε-caprolactone) (PCL) is a biodegradable and biocompatible polymer which has been extensively studied for medical applications. PCL is synthesized by ring opening polymerization (ROP) reaction of ε-caprolactone and the reaction can be catalyzed by lipase eznyme.

In this study, rice husk ash (RHA) was used as an inorganic support material for immobilization of free *Candida antarctica* lipase B (CALB), commercially known as Lipozyme®. RHA was chosen since it is an easily accessible and cheap material. Additionally, its high silica content makes RHA a suitable choice since it is known that silica is successfully used as a support material for enzyme immobilization [10, 11]. RHA was obtained by burning rice husks at 600 °C for 6 h. Then, surface modification was applied by using two different silanization agents, 3-aminopropyl trimethoxysilane (3-APTMS) and 3-glycidyloxypropyl trimethoxysilane (3-GPTMS). The modified RHA were used as support materials for immobilization of

CALB. CALB was immobilized by physical adsorption. Optimization studies were conducted by changing enzyme loading and silanization agent concentration. In addition, pH, temperature, storage and operational stabilities were measured, optimum pH and temperature were determined. The characterization of RHA, surface modified RHA and immobilized enzyme samples were done by Fourier transform infrared spectroscopy (FT-IR), thermal gravimetric analysis (TGA), scanning electron microscopy (SEM) and Brunauer-Emmett-Teller (BET) surface area analysis.

2. THEORETICAL STUDY

2.1 Enzyme Immobilization

Enzymes are very versatile catalysts and are able to catalyze very complex chemical processes under mild and environmentally safe conditions. For this reason, there is an upward trend in biocatalysis for industrial applications. However, there are limitations of enzyme utilization in industry due to some characteristic properties of enzymes. These features are generally low stability under non-physiological conditions, inhibition by the reaction products and solubility in water thus difficult recovery in aqueous media [6, 12, 13]. Enzyme immobilization is a technique to overcome these problems and adapt enzyme utilization for industrial applications. Enzyme immobilization is achieved by confining the enzyme molecules to a phase in which the substrates and the products are present and can be in contact. Immobilized enzymes have higher stability, catalytic activity and selectivity compared to free form and are easier to recover and reuse which allows enzymes to be used in continuous processes [13-15]. They also prevent protein contamination of the product [1, 4].

Although researches on enzyme immobilization dates back to 1940s, new techniques about this area started to develop after 1960s. In 1966, Chibata and coworkers reported the immobilization of Aspergillus oryzae aminoacylase for the resolution of synthetic racemic D - L amino acids, as being the first industrial use of immobilized eznymes [3].

In spite of many advantages of immobilization, there are also some disadvantages that are needed to be considered. For example, activity reduction due to repeated use is a major concern. Diffusion limitations may be caused by the restriction of enzyme and substrate mobility due to immobilization. Also, immobilization process creates additional cost since immobilization requires extra materials, mainly a support material [3, 16], which can be significantly reduced by adapting the use of cheap materials.

The properties of immobilized enzymes are dependent on both enzyme and support material properties. The functional groups of the enzyme determine the interaction between the enzyme and the support. Also, purity of the enzyme is a concern since impurities may decrease immobilization efficiency. pH and temperature stability of the enzyme also affects immobilized enzyme properties. The most important characteristic of the support material affecting immobilized enzyme properties is chemical structure. Physical resistance of the support material influences mechanical properties of the immobilized enzyme which is crucial for industrial applications [17].

2.1.1 Immobilization methods

There are several methods of enzyme immobilization including physical adsorption, entrapment, covalent attachment and cross-linking whose schematic representations can be seen in Figure 2.1 [18]. Among these methods, only physical adsorption is considered to be reversible, the others are irreversible applications [3].

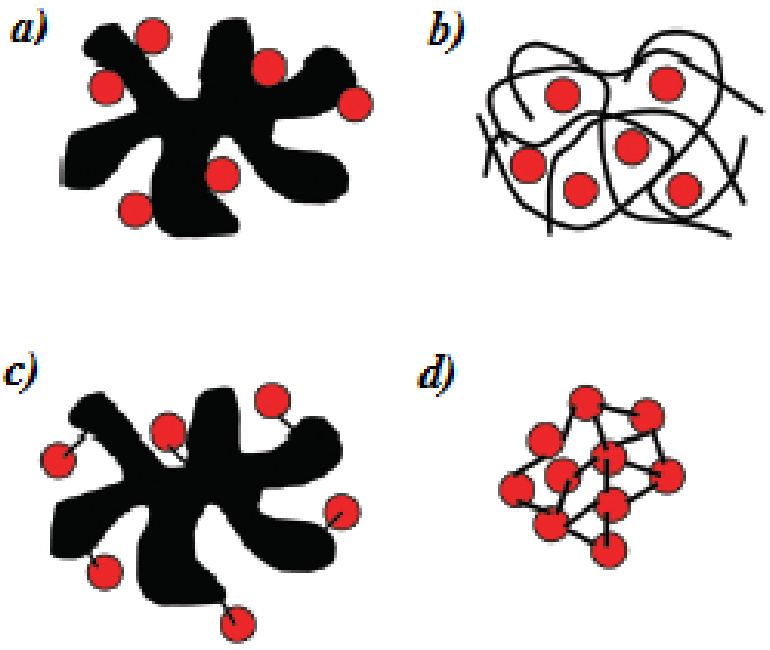

Figure 2.1: Enzyme immobilization methods, a) physical adsorption, b) entrapment, c) covalent attachment, d) cross-linking [18].

Physical adsorption is a simple and inexpensive immobilization method. The support material is brought into contact with the enzyme solution and adsorption occurs if the interactions between the enzyme and the support material are strong enough. In this method, enzyme attaches to the support material by weak forces such as Van der Waals, hydrophobic or ionic interactions [17, 19]. In case of lipase enzyme, generally hydrophobic interactions cause adsorption [18]. Adsorption is often preferred since it is a simple and low-cost procedure. Additionally, it is reversible allowing support material to be recovered and reused. There is no need for any chemical addition to the process which eliminates the risk of undesired modification of the enzyme and high lipase activity recovery can be achieved [7, 19]. However, in case of adsorption the major disadvantage is that enzyme tends to leach out especially in aqueous media [7, 17]. For immobilization of lipase enzyme, physical adsorption is the best choice for use in organic media. Various types of supports are used for lipase immobilization via physical adsorption, such as polymers, carbon nanofibers or silica [17].

Entrapment is achieved by capturing the enzyme within a polymer matrix in which mobility of the enzyme is merely restricted [1, 19]. Enzyme inclusion prevents leakage and direct contact with the environment thus protecting the enzyme from the adverse effect of the conditions of the environment. Most commonly used systems for entrapment are silica sol-gel matrices. Silica sol-gel matrices are formed through hydrolytic polymerization. These silica matrices are produced by silane hydrolysis with acid or base catalysis. Entrapment in sol-gel materials is found to be applicable to wide variety of lipases. They yield high activity in organic media. This might be because lipase high disperses in the sol-gel matrix [17]. There are also entrapment polymers which are not silica-based such as epoxide activated hydrogels or poly(vinyl alcohol) (PVA). However, mass transfer limitations and low enzyme loading are the disadvantages of this method [7].

In covalent attachment, covalent bond formation occurs through functional groups of the enzyme such as amino groups, carboxyl groups, hydroxyl groups or sulfhydryl groups [20]. It provides strong binding between the support and the enzyme thus reuse capacity of the covalently immobilized enzyme is higher than physically immobilized enzyme [21]. There are two major methods of covalent attachment (shown in Figure 2.2): covalent attachment via long spacer arm and covalent

attachment via multipoint attachment. Multipoint attachment has higher stability whereas in covalent attachment via spacer arm, restriction of enzyme configuration is less [17]. Support materials with epoxide groups are generally preferred for covalent attachment since epoxide groups and amine groups are reactive and form stable bond under mild conditions [7]. However, catalytic sites of the enzyme should not be involved in covalent bonding in order to obtain high activity. Covalent attachment is preferred when product purity is a concern [3].

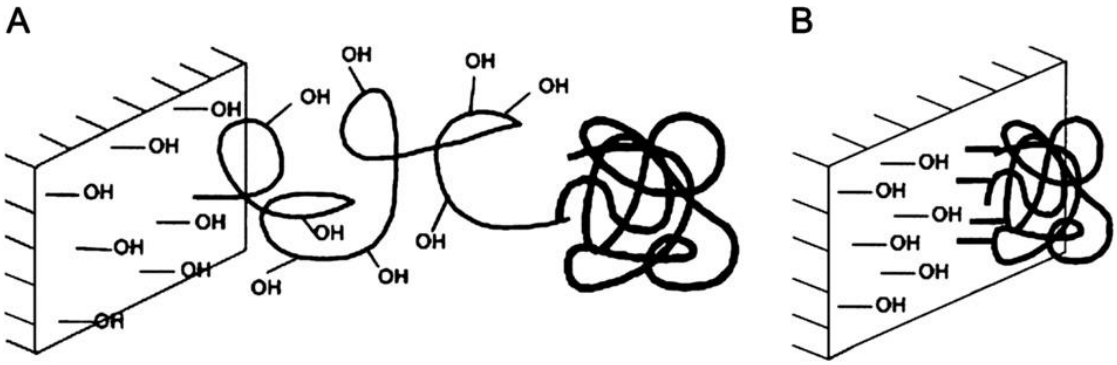

Figure 2.2: Immobilization through covalent attachment (A) via long spacer arm (B) via multipoint attachment [17].

Immobilization by cross-linking is the formation of intermolecular cross-linkages. It is achieved by the addition of a multifunctional reagent such as glutaraldehyde. Cross-linking may not requiere a support material to which the enzyme will be bound. There are several systems that do not involve a support material: cross-linked dissolved enzymes (CLEs), cross-linked enzyme crystals (CLECs), cross-linked enzyme aggregates (CLEAs), cross-linked spray-dried enzyme (CLSDs). CLEs and CLECs were discovered in the 1960s, by enzyme cross-linkage with a bifunctional cross-linker and by crystalline enzyme cross-linking with glutaraldehyde, respectively. CLEs have some disadvantages such as low mechanical stability and low activity. On the other hand, CLECs are very active and stable immobilized enzymes. They are easily recovered and reused thuse they are suitable for industrial applications. However, in order to carry out this technique, highly purified crystallisable enzymes must be used which is costly [17]. Another type of supportless immobilization via cross-linking is spherezyme. It is prepared by forming a water-in-oil emulsion of dissolved lipase and addition of a cross-linking agent. The lipase produced by this method is active in both aqueous and organic media [7]. The comparison of support-free cross-linking methods is shown in Figure 2.3.

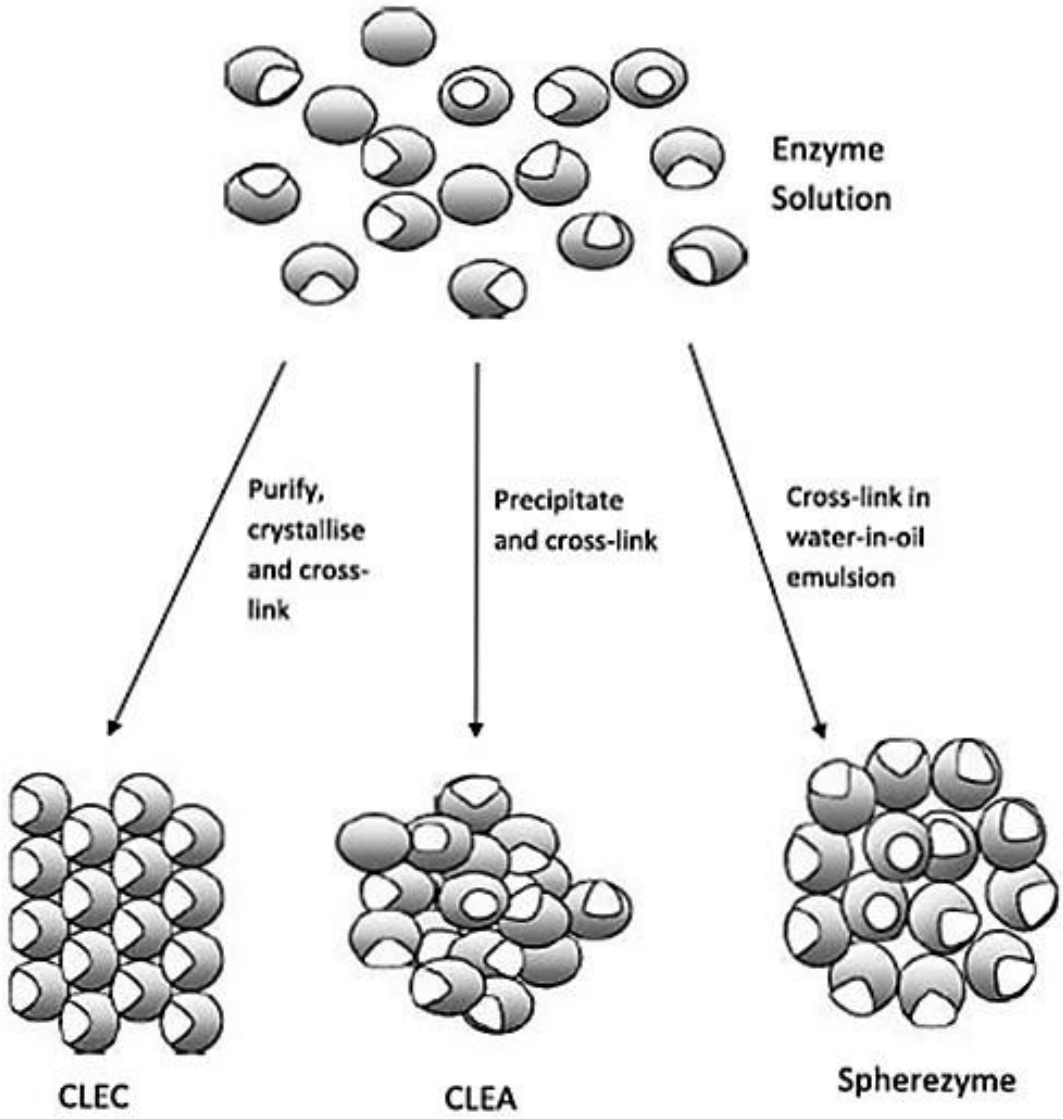

Figure 2.3: Comparison of support-free cross-linking methods [7].

2.1.2 Support materials

It was mentioned earlier that immobilization process requires a support material. Selection of support material is an important matter in enzyme immobilization since support material is involved in determination of immobilized enzyme properties. Chemical structure, physical and mechanical properties and pore size of the support material are considered during selection [17, 22, 23]. The functional groups on the surface of the support material determines the interactions between the support material and the enzyme during immobilization. Thus the chemical sturcture of the support material is an important property that affects the properties of the immobilized enzyme. Furthermore, if the pore size of the support material is small,

the activity of the immobilized enzyme may be low due to diffusional limitations. On the other hand, support materials with large pore sizes may cause enzymes to cluster and thus causes low activity. Mechanical properties should be compatible with the reactor type in which the immobilized enzyme will be used. An immobilized enzyme, which will be used in a stirred tank, should be resistant to abrasion, while an immobilized enzyme, which will be used in a column, should have flow resistance [17]. Furthermore, a suitable support material should have some characteristics; such as resistance to physical forces and microbial contamination, ease of access and low cost [3].

Support materials are basically divided into two groups: organic support materials and inorganic support materials. Organic support materials can be natural or synthetic. Organic support materials obtained from natural sources, mostly polymers, show good compatibility with enzymes. Since they are hydrophilic, they can only involved in weak bindings thus the mechanical stability of these materials should be improved [17]. Some natural polymers used as support materials are polysaccharides and (cellulose, alginate, agarose, starch, chitosan and chitin), proteins (collagen, albumin). Synthetic polymers can also be used as support materials such as polystyrene, polyacrylate, polymethacrylates, polyacrylamide and polyamides [3]. Agarose is a support material that is extensively used. Due to its high porosity, its capacity to bind proteins is high. Additionally, it is commercially available material. However, the major disadvantage of this material is its high cost [3]. Acrylic resins such as Eupergit® C is another widely used support material. It is a macroporous copolymer which is very hydrophilic and have a good pH stability. Though it has attractive properties, it has a major disadvantage which is diffusional limitations [17].

On the other hand, inorganic supports have better stability compared to organic support materials. However, abrasion may occur due to physical forces during reactions [17]. Silica and silica derivatives, metals, bentonite and celite are some widely studied inorganic support materials [3, 17]. Silica-based materials and other inorganic support materials have small pore sizes, around 8 nm in diameter, can only allow small-sized enzymes to enter the pores; leaving larger enzymes on he surface of the support material [22]. For both organic and inorganic support, there have been extensive studies in the literature by using different enzymes and different immobilization techniques [9].

2.1.3 Surface modification by silanization

Silanization is a widely used technique for surface modification of materials. Silanization agents are used for this process. Organosilanes are widely used for surface modification of hydroxylated (silanol for silica-based materials) surfaces where hydroxyl groups on the surface react with active groups of the silanization agent, generally alkoxy groups. Trifunctional silanes have higher reactivity and monolayers of trifunctional silanes are believed to yield well-ordered and compact films [24, 25]. Most widely used trifunctional organosilanes are 3-aminopropyl triethoxysilane (3-APTES), 3-aminopropyl trimethoxysilane (3-APTMS) and 3-glycidyloxypropyl trimethoxysilane (3-GPTMS).

These agents react with the surface silanol groups through their alkoxy groups, which are methoxy for 3-APTMS and 3-GPTMS and ethoxy for 3-APTES. After silanization process, depending on the type of silanization agent amine (NH_2) or epoxy ring are obtained on the surface. Amine groups of APTES and APTMS are primary amines. Silanization mechanisms of these three silanization agents are shown in Figure 2.4 [26]. As can be seen in the figure, siloxane bonds are formed in betwwen the silica-based support material and the silanization agent through the alkoxy groups of the silanization agents.

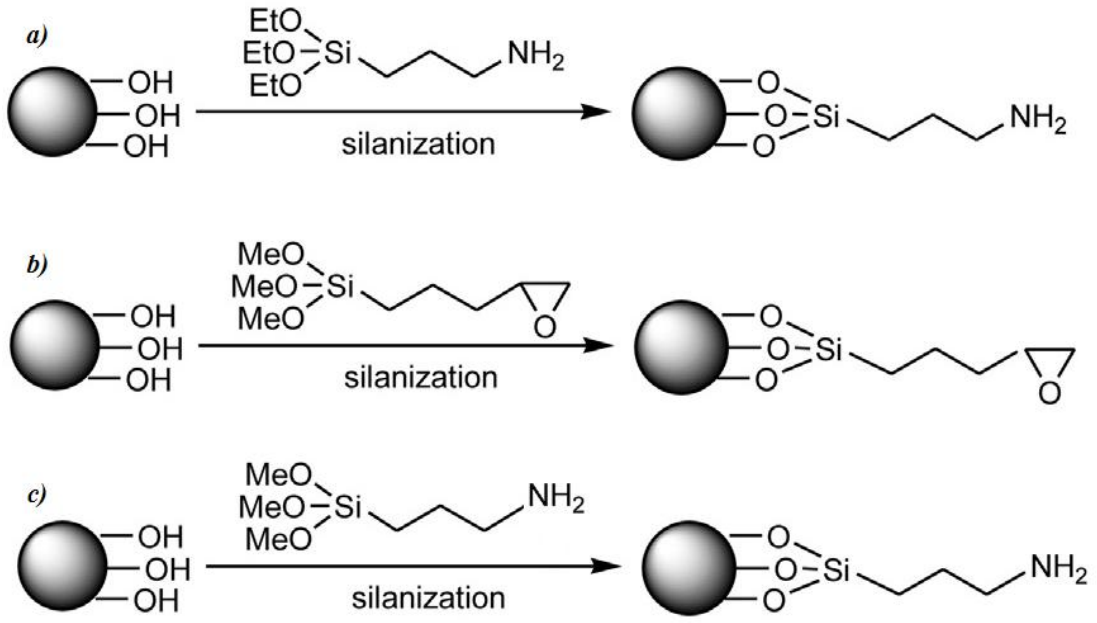

Figure 2.4: Silanization by a) 3-APTES b) 3-GPTMS c) 3-APTMS [26].

Due to hydrophobic nature of these silanization agents, silanization processes utilizing these agents are conducted in organic solvents which may create some problems such as high cost, pollution, low efficiency and solvent recovery, which are needed to be solved for mass production [27].

After silanization process, enzyme can be covalently immobilized onto the modified support material. However, in covalent bonding enzyme loading capacity is low since enzyme is immobilized as monolayers [28]. An alternative to covalent bonding, with higher enzyme loading capacity, is crosslinking. For amine-functional silanization agents (3-APTES, 3-APTMS), in order to form crosslinkages between the functinalized support material and the enzyme, an agent (crosslinking agent) should be used. Most widely used crosslink agent is glutaraldehyde. The mechanism of this process by using 3-APTES is given in Figure 2.5 [26].

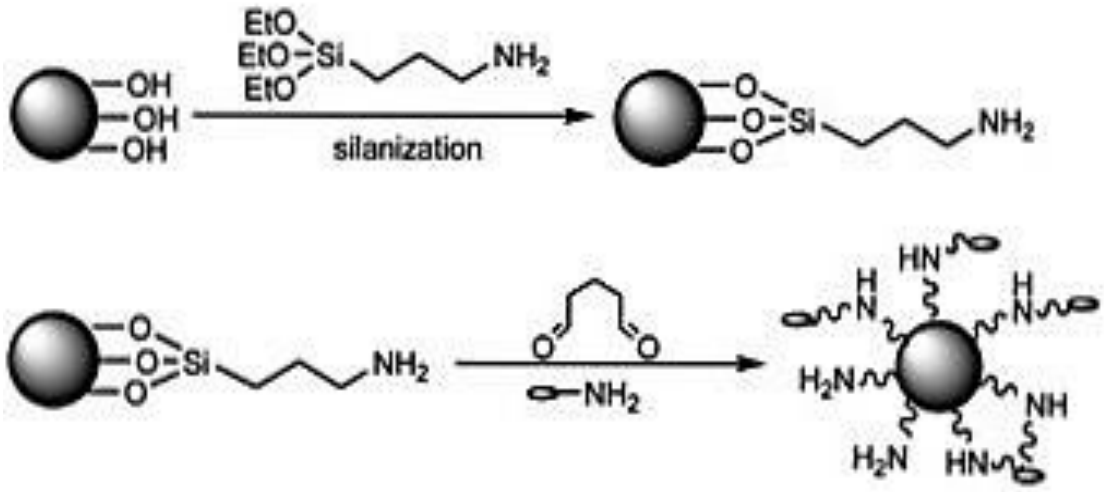

Figure 2.5: Mechanism of immobilization on 3-APTES-modified material [26].

For the case of 3-GPTMS-modification, enzyme immobilization occurs through covalent bonding without any requirement for additional chemical agent or reaction step since epoxy ring is reactive with the nucleophiles of the enzyme under mild conditions. The bonding between epoxy ring and the nucleophiles such as amine, hydroxyl and thiol groups is very strong and stable. Thus, epoxy-activated surfaces are very attractive materials for enzyme immobilization [29]. The mechanism can be seen in Figure 2.6 [26].

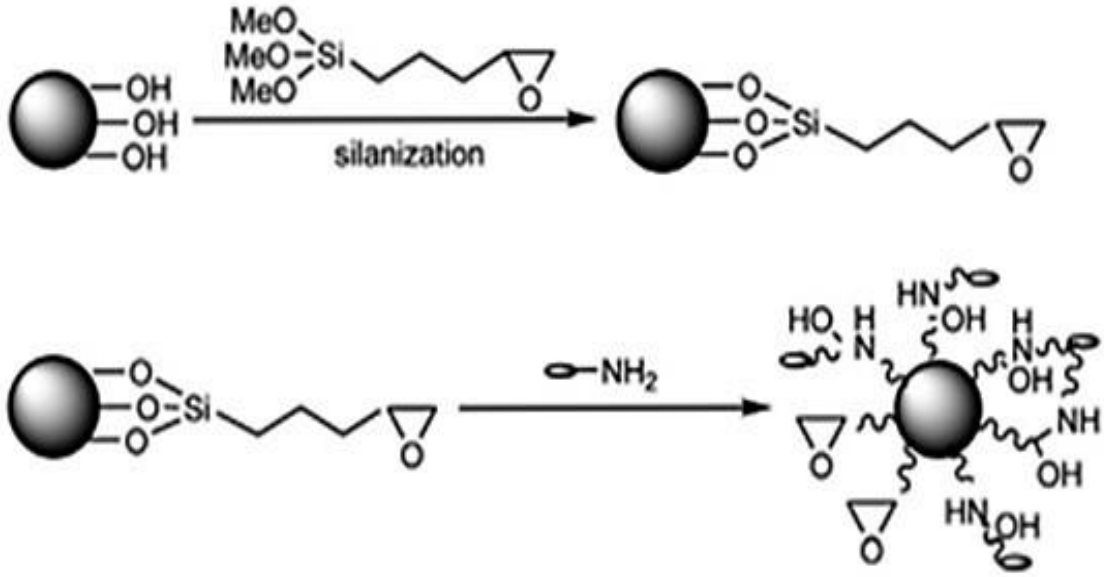

Figure 2.6: Mechanism of immobilization on 3-GPTMS-modified material [26].

A three-step immobilization method by using amino-epoxy heterofunctional support material is given in literature which is also shown in Figure 2.7. Such support material are prepared by using activated amino-supports and butanediol diglycidyl ether and at the end of the procedure both amino groups and epoxy groups are obtained on the surface of the support material. A commercially available amino-epoxy heterofunctional support material is known as Resindion [6]. For easy modification with epoxy groups, amino groups on the support material should have pK around 9-10. Later they become ionized as secondary amine groups to allow physical adsorption of the enzyme. The first step of this method is physical adsorption at pH 7.0, and the physical interactions occur between the amino groups of the support material and amino groups of the enzyme. At the second step covalent attachment occurs between epoxy and amino groups. This reaction also takes places in a medium with pH 7.0. Finally as the third step multipoint covalent attachment between the epoxy groups of the support material and the amino groups of the enzyme is achieved under basic conditions (pH 10.0). At the end of this procedure, the enzyme is covalently attached to a heterofunctional (amino groups and epoxy groups) support material [6].

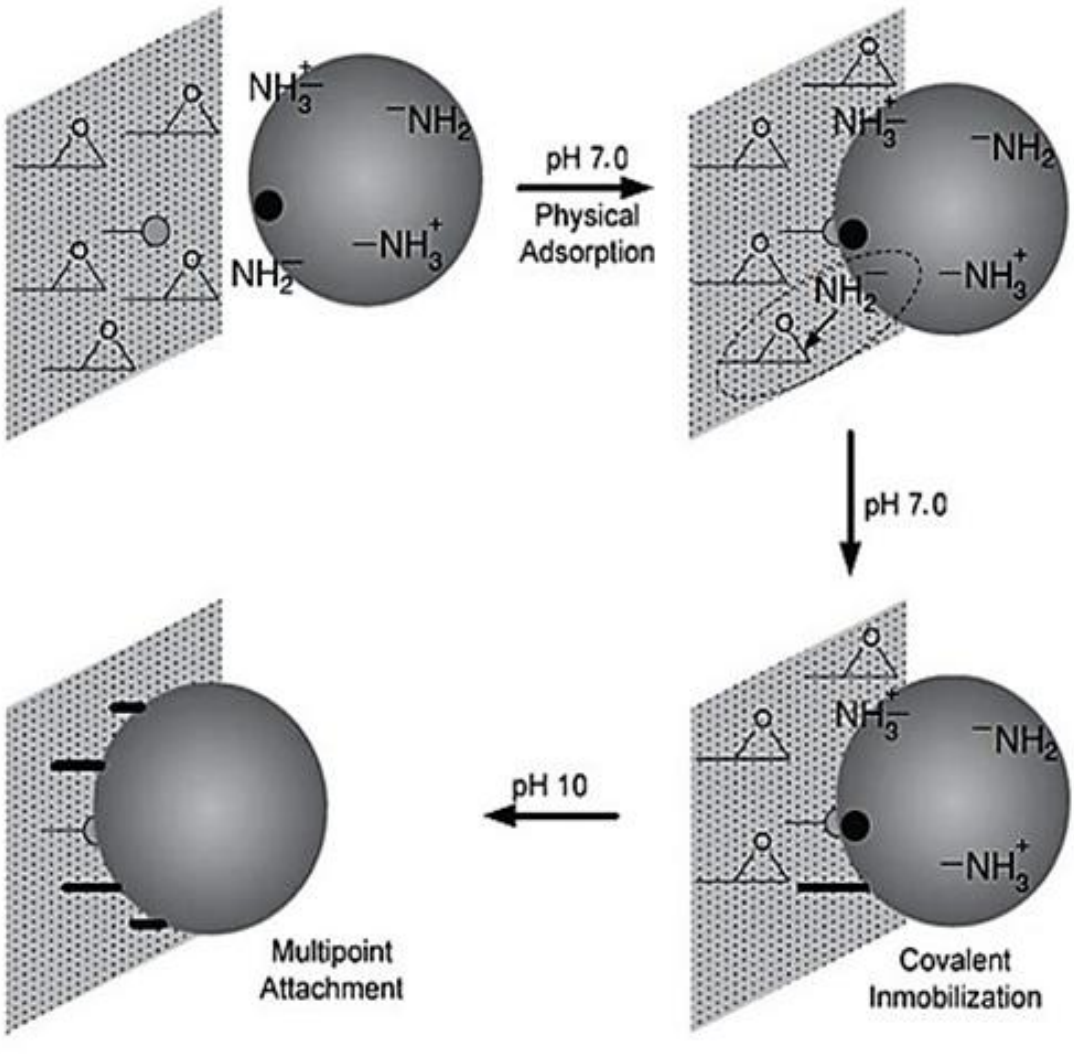

Figure 2.7: Three-step enzyme immobilization on an epoxy-activated support [6].

2.2 Lipase Enzyme

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are defined as enzymes which have capability of hydrolyzing carboxyl esters of long-chain acylglycerol. However, they accept a wide variety of substrates. They also have catalytic activity on hydrolysis of triglycerides yielding fatty acids and glycerol, esterification and transesterification reactions. Lipases are characterized by an α/β hydrolase fold and disulfide bridges which make enzyme stable [30].

The active site of lipases is a catalytic triad composed of serine (Ser), aspartic acid (Asp) and histidine (His) [6]. As all enzymes, lipases undergo severe conformational changes during catalysis. In the presence of a hydrophobic interface, the active site of the enzyme is exposed to the hydrophobic phase and is open to substrate access. This

conformational structure is called “open form”. An α-helicoidal polypeptide chain (lid) covers the active site of the enzyme hindering the interaction between the active site and the substrate if there is not a hydrophobic interface. This structure is called the “closed form” of the enzyme. This phenomenon is known as interfacial activation [18]. The two conformational structures of lipase are given in Figure 2.8 [5].

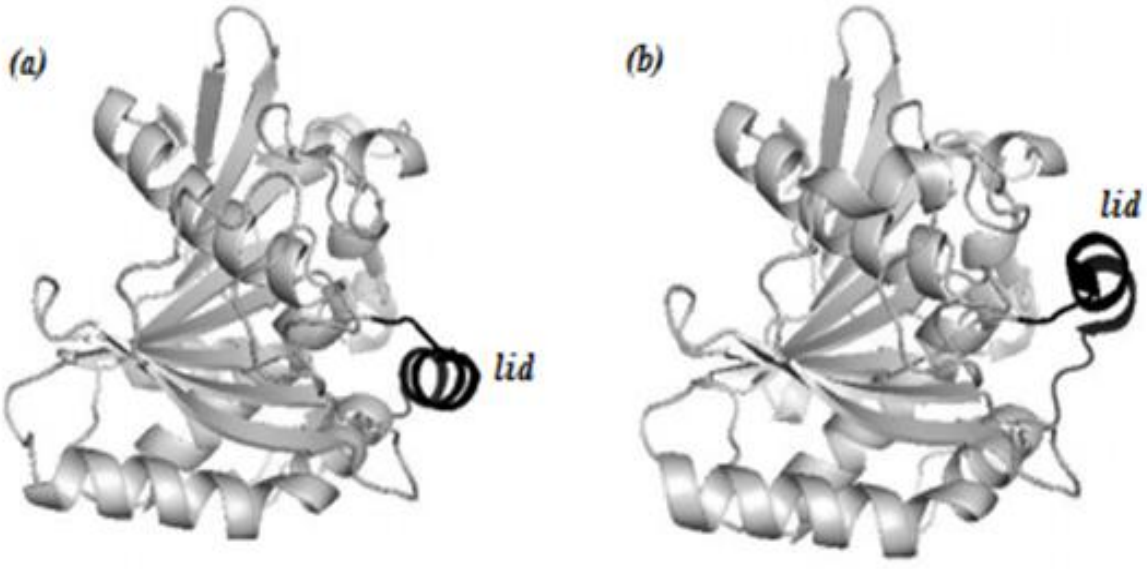

Figure 2.8: (a) open and (b) closed forms of lipase [5].

The selectivity of lipase enzyme can be considered in terms of three types of selectivity: regioselectivity (the selectivity towards the direction), enantioselectivity (the selectivity towards one of a pair of enantiomers) and chemoselectivity (the selectivity towards functional groups). Due to their selectivity, being stable in organic solvents and being versatile, lipases are important for industrial applications [30]. These properties also allow development of lipase-catalyzed polymerization reactions. It is reported that lipases can catalyze ring opening polymerization (ROP) of cyclic monomers, polymerization of diacid derivatives and glycols and polycondesation of oxyacid derivatives [31].

Lipase enzyme can be obtained from a variety of organisms for various applications which are summarized in Table 2.1 [30]. These organisms can be fungus, bacteria or mammals. Most of the lipases are obtained from fungal or bacterial organisms. Dog, horse, rat, Guinea pig, porcupine pancreatic lipase are some lipase enzymes which are obtained from mammals.

Table 2.1: Lipases from different organisms [30].

Type of Organism	Lipase
Fungus	*Thermomyces lanuginosus, Rhizopus oryzae, Rhizopus niveus, Candida antarctica, Candida rugosa, Geotrichum candidum, Penicillium camembertii, Penicillium expansum, Yarrowia lipolytica* lipase
Bacteria	*Bacillus subtilis, Pseudomonas sp., P. aeruginosa, P. cepacia, P. glumae, Chromobacterium viscosum, Geobacillus thermocatenulatus, Geobacillus stearothermophilus, G. zalihae, Photobacterium sp., S. marcescens, Staphylococcus hyicus, Streptomyces exfoliates* lipase
Mammal	Dog, horse, rat, Guinea pig, porcupine pancreatic lipase

For polymer synthesis, *Candida antarctica* lipase B (CALB) provides the most efficient catalysis. The molecular weight of CALB is 33kD with 317 amino acid residue and has an isoelectric point (pI) of 6.0. The active site of CALB is a catalytic triad of Ser105-His224-Asp187 [32]. The commercially available CALB, Novozyme® 435, is physically adsorbed on a macroporous acrylic polymer resin [33] and is used for ring opening polymerization of several lactones. Even though it has many advantages in polymer synthesis, there are some major problems of utilization of Novozyme 435: high cost, leaching problem of the enzyme and diffusional limitations [20]. Several studies have been conducted in order to find solutions for these problems [11, 14, 34, 35].

2.3 Rice Husk Ash (RHA) as a Support Material

Rice husk is a by-product of rice production process which constitutes 20 % of rice by weight. Its chemical composition is 50 % cellulose, 25-30 % lignin and 15-20 % silica [36]. Rice husk ash is obtained by burning of rice husk. During burning, cellulose and lignin are removed leaving silica ash behind. RHA is a light and highly porous material with high silica content. It constitutes about 20% of the husk mass and 4% of rice in husk. Rice husks find various application areas such as production of mesoporous sieves, insulating materials, fertilizers, silicon carbide, cement as additive and waste water treatment due to its high absorptivity [36, 37]. The properties of ash may depend on the conditions of the burning process. Structure of

the silica content may vary with combustion temperature: formation of amorphous silica ash at 550-800 °C and formation of crystalline silica ash at higher temperatures [38]. It is vital to control the combustion conditions in order to produce ash for the desired end use since different crystalline structures have different properties.

In the literature, it is reported that high content of amorphous silica (95 %) can be obtained by burning at 700 °C for 6 h [37, 39, 40]. Thus it can be concluded that RHA is a porous support material suitable for enzyme immobilization. Additionally, consumption of RHA is an advantageous application since disposal of this ash has harmful impacts on environment [41]. Rice husks and rice husk ash can be seen in Figure 2.9 [37].

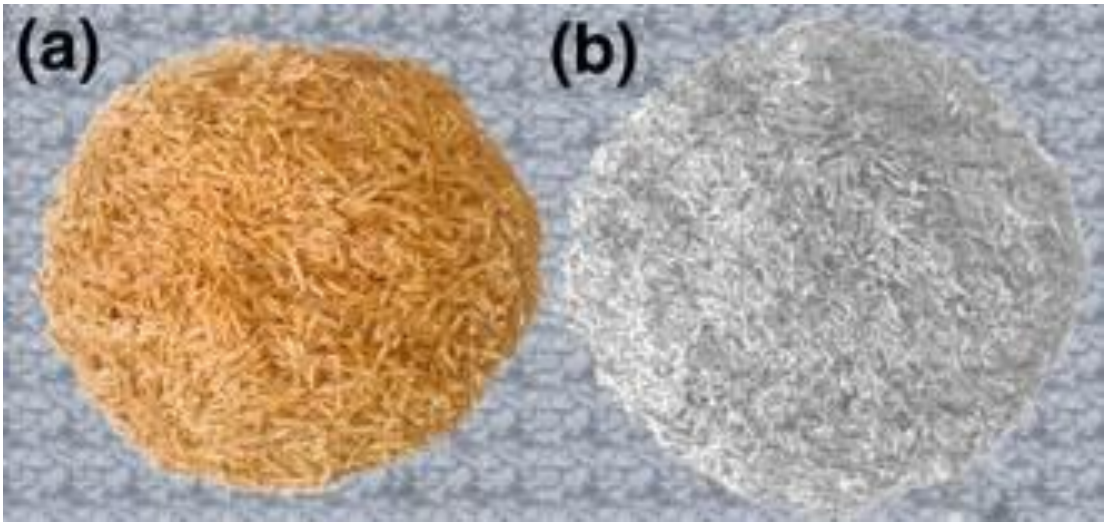

Figure 2.9: (a) Rice husk (b) Rice husk ash [37].

2.4 Applications of Lipases

Lipases are used in many industrial areas such as detergent, pulp and paper, energy and food industries, production of fine chemcials, cosmetics and pharmaceuticals, medical applications and polymer synthesis [30].

Lipases have hydrolytic activity on fats thus can be used as additives in detergents, responsible for removal of oil stains. However, the lipase enzyme to be used in detergents should be able to operate under washing conditions which can be considered harsh for enzymes (high pH and temperature). Additionally, it should be resistant to the damages of surfactants in the detergent which are one of the major ingredients of the detergent [42].

In pulp and paper industry, lipases are used for removal of pitch. Pitch is the hydrophobic components of wood, mostly waxes and triglycerides. Its deposition reduces the quality of pulp and thus is not desired. Utilization of lipases in pulp and paper industry is an advance technology [6]. It also helps to control the contaminants in the waste water [30]. A paper company in Japan has developed a system to utilize *Candida rugosa* lipase and achieved to hydrolize 90 % of the wood triglycerides [42].

Lipases are used to replace the conventionally used emulsifiers in food industry [43]. In dairy products, especially in chesee production, lipases are responsible for flavor development by selective hydrolysis of triglycerides [6]. They can also change the properties of lipids by creating some structural alterations. This property is used to produce high valued fats in food industry. One example of this application is the utilization of immobilized Rhizomucor miehei in order to replace palmitic acid with stearic acid in palm oil [42].

In energy industry, lipases can be mainly used for biodiesel production. It is an important application since it is vital to replace fossil fuels with renewable energy sources and come up with solutions for CO_2 build-up in the atmosphere [6]. Biodiesel is a mixture of fatty acid alkyl esters and is produced by transesterification of oil [44]. Since it is sulfur free and reduce the emission of hydrocarbons and CO, biodiesels provide an eco-friendly and clean alternative to conventional fossil fuels [45]. Ezymatic processes, rather than conventional processing using chemical catalysts, are preferred since enzymes can be operated under milder conditions and do not produce any chemical wastes. However, lipase catalyzed reactions are still not very commonly used due to the high cost of lipase enzyme [46]. Immobilized enzymes can be a solution for this problem and it is shown that immobilized lipases gives better results compared to free lipases [47]. There have been an extensive amount of researches about this area [45, 48 - 51].

Biopolymers are generally preferred for medical application since they are biodegradable and biocompatible with human body thus nontoxic. Poly (ε-caprolactone) (PCL) is a biodegradable and biocompatible polymer which has been extensively studied for medical applications. PCL is synthesized by ring opening polymerization (ROP) reaction of ε-caprolactone and the reaction can be catalyzed by lipase eznyme [52]. Immobilized CALB is very efficient poly (ε-caprolactone)

synthesis; indeed Novozyme 435® is efficient for poly (ε-caprolactone) synthesis [53]. Various studies aiming to improve the performance of lipase catalysis by using different immobilization methods or different support materials exist in the literarure. In one study, a silica-based material was used as a support material for CALB immobilization by physical adsorption, and the results show that this immobilized CALB can be good alternative for poly (ε-caprolactone) synthesis [54].

3. MATERIALS AND METHODS

3.1 Materials

Free CALB (CALB L, Lipozyme®) was immobilized onto surface-modified rice husk ash and purchased from Novozymes. Rice husk ash (RHA) was used as an immobilization support material and was obtained by burning rice husks which were supplied by a rice production company in Edirne, Turkey. (3-Glycidyloxypropyl) trimethoxysilane (3-GPTMS) (98%, $C_9H_{20}O_5Si$) and (3-Aminopropyl)trimethoxysilane (3-APTMS) (95%, $C_6H_{17}O_3NSi$), silanization agents for surface modification of support material, were purchased from Aldrich and Acros, respectively. 99 % pure acetone (C_3H_6O) was purchased from Riedel-de Häen and used as a solvent for 3-GPTMS and 3-APTMS modification. Phosphate buffer solutions were used during immobilization processes. These solutions were prepared with monobasic sodium phosphate ($NaH_2PO_4.H_2O$) and dibasic sodium phosphate ($Na_2HPO_4.7H_2O$) salts which were purchased from Carlo Erba and Merck, respectively. Olive oil from Komili was used in activity measurement of lipase enzyme. Ethanol (99%, C_2H_5OH) purchased from Merck. It was used to terminate hydrolysis reaction during activity measurement. As an indicator, phenolphthalein (1% in ethanol) was used in titration during lipase activity measurement and purchased from Merck.

3.2 Methods

3.2.1. Support material

Rice husks were washed with distilled water and dried in an oven (Electro mag M3025P) for 24 h. Then the dried rice husks were burned in a furnace at 600 °C for 6 h in order to obtain RHA. The temperature of the furnace was increased stepwise (10 °C/min) until the burning temperature was reached. At the end of this procedure, obtained RHA was stored in a desiccator for further use. RHA obtained at the end of this procedure can be seen in Figure 3.1.

Figure 3.1: RHA obtained by burning rice husk.

RHA was modified with a silanization agent before it was used as a support material for lipase immobilization. For this purpose, 250 mg RHA was mixed with 15 % (v/v) 3-GPTMS in 5 ml acetone in test tubes. The test tubes were placed in a shaking water bath (Julabo SW22) for incubation. The incubation was performed at 50 °C and 160 rpm for 2 h. The same procedure was applied by using 3-APTMS as silanization agent.

After incubation, surface modified RHA was filtered and washed with distilled water under vacuum created by using a pump (Sartorius stedim 16612). The filtered RHA was put in oven at 60 °C for 4 h. The activated RHA was stored in a desiccator for further use in lipase immobilization as support material. The silane-modified RHA samples are shown in Figure 3.2.

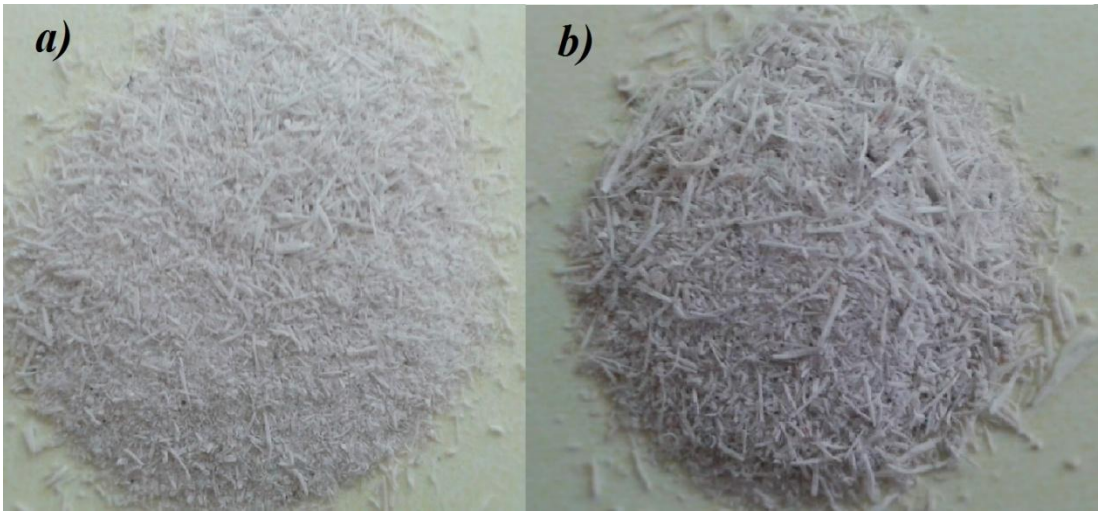

Figure 3.2 : Modified RHA with a) 3-GPTMS b) 3-APTMS.

3.2.2. Lipase immobilization

Free form of lipase enzyme was immobilized on surface-modified RHA by physical adsorption. The surface-modified RHA was mixed with free lipase enzyme (enzyme to support ratio of 2 µl/mg) and 25 ml 0.015 M, pH 7.0 phosphate buffer solution on a magnetic stirrer at room temperature for 5 h. At the end of mixing, immobilized enzyme samples were collected by filtrating the solution using a vacuum pump. The first filtrate was taken into a graduated cylinder before washing the enzyme samples in order to use protein determination measurements. After the first filtrate was collected, enzyme samples were washed with 0.015 M, pH 7.0 phosphate buffer solution. Then they were placed in the oven at 30 °C and dried for 12 h. Dry immobilized enzyme samples, which are shown in Figure 3.3, were stored in fridge at 4 °C.

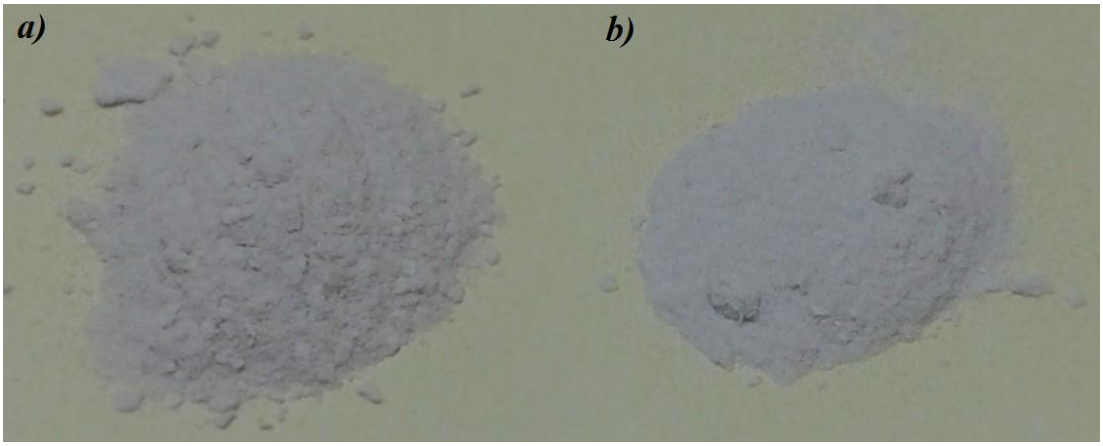

Figure 3.3 : Immobilized enzyme on a) 3-GPTMS-modified RHA b) 3-APTMS-modified RHA.

In addition, parametric experiments were conducted in advance in order to observe the effects of silanization agent concentration and enzyme loading ratio and determine the optimum conditions. Surface modification was carried out with five different concentrations of 3-GPTMS and 3-APTMS (5%, 10 %, 15 %, 20 % and 30 % (v/v)) in 5 ml acetone. The same immobilization procedure was applied to the support materials that were modified with different silanization agent concentration. Four different ratios were used: 0.5 µl/mg, 1 µl/mg, 2 µl/mg and 3 µl/mg.

Phosphate buffer solution (0.015 M, pH 7.0) was prepared mixing 29.25 ml of $NaH_2PO_4.H_2O$ solution and 45.75 ml of $Na_2HPO_4.7H_2O$ solution and completing the mixture to 1 l with distilled water.

3.2.3. Protein assay

The amount of lipase enzyme immobilized on the support was determined by measuring the protein content of the first filtrate obtained from the immobilization process. Protein determination was conducted with UV spectrophotometric method at 280 nm. This method is based on the presence of tyrosine and tryptophan amino acids which absorb ultraviolet light strongly at 280 nm. The blank solution was phospahte buffer solution (0.015 M, pH 7.0) in which the samples were prepared. A standard curve was drawn earlier and used to determine the protein concentration of samples. The standard curve is given in the Appendices section.

Immobilization efficiency is the percentage of the ratio between the amount of protein immobilized onto the support material and the amount of protein loaded and was calculated by Equation 3.1 [55].

$$Immobilization\ efficiency = \frac{amount\ \ of\ protein\ \ immobilized}{amount\ \ of\ protein\ \ loaded} \times 100 \qquad (3.1)$$

3.2.4. Activity measurement of immobilized enzymes

Activity measurements of the immobilized enzymes were conducted with titration method. This method depends on the hydrolytic activity of lipase on oils and the measurements depended on the hydrolysis rate of olive oil [56]. Enzyme samples were dispersed in phosphate buffer solution (0.015 M, pH 7.0; concentration of 10 mg/ml). Then, 100 µl of the dispersed enzyme solution was mixed with 500 µl olive oil in a test tube. 2.5 ml phosphate buffer solution (0.1 M, pH 7.2) was added to the mixture in order to neutralize the reaction medium. The reaction mixture was incubated in the shaking water bath at 37 °C and 120 rpm for 30 min. The reaction was terminated by adding 1.25 ml acetone and 1.25 ml ethanol to the mixture. A few drops of phenolphthalein were also added in order to be able to observe a color change. The mixture was titrated with 0.1 M NaOH solution. One unit of enzyme activity (U) is defined as 1µmol of fatty acid released during the reaction, which is also equivalent to the amount of NaOH spent during titration, per minute. Specific activity (U/mg) is the ratio of immobilized enzyme activity to amount of immobilized protein [55]. Phosphate buffer solution (0.1 M, pH 7.2) was prepared by dissolving 0.4363 g $NaH_2PO_4.H_2O$ and 1.8326 g $Na_2HPO_4.7H_2O$ in 100 ml of distilled water.

3.2.5. Storage and operational stability of immobilized enzymes

Storage and operational stabilities of an enzyme are important parameters that need to be optimized for industrial and large scale applications. For storage stability determination, activity of the immobilized enzyme samples was measured every 15 days for 3 months. Enzyme samples were stored at 4 °C in between.

Operational stability experiments were conducted by using immobilized enzyme samples repeatedly. 10 mg of immobilized enzyme was mixed with 1000 µl phosphate buffer solution (0.015 M, pH 7.0) and 5000 µl olive oil in centrifuge tubes. The mixtures were incubated at 37 °C and 120 rpm for 30 min. After incubation, the mixtures were centrifuged at 6000 rpm for 15 min. Liquid phase was separated for activity measurement which was explained earlier. Solid phase was taken and the procedure was repeated.

3.2.6. Determination of optimum pH and temperature

For determination of optimum pH, buffer solutions were prepared with different pH (pH 4.5, 5.5, 6.5, 8.5, 9.5, 10.5). 10 mg of enzyme samples were dispersed in 1 ml of these buffer solutions, instead of pH 7.0 phosphate buffer solution. 100 µl of these dispersed solutions were mixed with 500 µl olive oil. The mixtures were incubated at 37 °C and 120 rpm for 30 min. 2.5 ml phosphate buffer solution (0.1 M, pH 7.2) was added to the incubated mixtures. The activities of the samples were measured.

Optimum temperature was determined by simply changing the incubation temperature and applying the same activity measurement procedure. Six different temperatures were tried: 30 °C, 40 °C, 45 °C, 50 °C, 55 °C and 60 °C.

3.2.7. Determination of pH and temperature stability

In order to determine the stable region that immobilized enzymes work, pH and temperature stability measurements were done. Different buffer solutions with different pH values (pH 4.5, 5.5, 6.5, 7.2, 8.5, 9.5, 10.5) were used in pH stability measurements. 10 mg/ml enzyme solution was prepared by using pH 7.0 phosphate buffer solution. 1 ml enzyme solution and 1ml buffer solution with different pH values were mixed in test tubes and stored at 37 °C for 24 h. After 24 h was over, 100 µl of these solutions were mixed with 500 µl olive oil. The rest of the experiments were conducted as given in the previous section.

Temperature stability measurements were carried out at different temperatures (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C). Firstly, 10 mg/ml enzyme solution was prepared in 1ml pH 7.0 phosphate buffer solution. 500 µl of these solutions were taken into test tubes and kept at the experiment temperatures for 15 min. Then, the samples were kept at room temperature for 5 min. After that, the same activity measurement procedure was applied as in pH stability experiment.

3.3 Characterization Techniques

3.2.1. Ultraviolet (UV) spectrophotometry

Immobilization efficiency of lipase enzyme was calculated by measuring absorbance values with UV mini 1240 SHIMADZU spectrophotometer. Phosphate buffer solution (0.015 M, pH 7.0) was the blank sample and the samples were prepared in this phosphate buffer solution. The measurements were recorded at 280 nm.

3.2.2. Fourier transform infrared spectroscopy (FT-IR)

Chemical structures and compositions of RHA, surface-modified RHA and immobilized enzymes were determined by FTIR analysis. Changes in the chemical structure of support material during the silanization and immobilization processes can be tracked through the peaks obtained from FTIR spectra.

3.2.3. Thermal gravimetric analysis (TGA)

Thermal gravimetric analysis was carried out for thermal characterization of surface modified RHA. Analysis was conducted with SEIKO TG/DTA 6300. 5-10 mg of samples were heated to 1000 °C with a rate of 10 °C/min and cumulative weight loss of samples were recorded.

3.2.4. Scanning electron microscopy (SEM)

Scanning electron microscopy (JEOL JSM-6390LV SEM) was used in order to observe the surface morphologies of RHA, surface-modified RHA and immobilized enzyme samples. The samples were coated with platinum before scanning since the samples were not conductive. Scanning was performed at 5 kV with x1000 magnification.

3.2.5. Brunauer-Emmett-Teller Surface Area Analysis (BET)

Nitrogen isotherms were measured at the temperature of liquid nitrogen (N_2) with a Micromeritics ASAP 2010 apparatus. Liquid N_2 used during analysis was at 77.30 K. The surface areas of RHA and were determined by the BET method. The total pore volume was calculated from the amount of vapor adsorbed at a relative pressure (P/P_0), where P and P_0 are the measured and equilibrium pressures, respectively.

4. RESULTS AND DISCUSSION

4.1 Surface Modification of RHA

As the first step of immobilization process, RHA surface was modified by silanization method. Two different silanization agents, 3-APTMS and 3-GPTMS, were used for this purpose. The modified RHA samples were characterized by FT-IR spectroscopy.

Two sharp peaks can be observed in the FT-IR analysis of 3-GPTMS-modified RHA which is given in Figure 4.1. The first one is at approximately 800 cm^{-1} which corresponds to Si-CH_3 rocking [57]. This region is also a characteristic peak region for epoxy ring of 3-GPTMS [27] which has epoxy ring on its surface as functional group. Thus, it can be concluded that RHA silanization with 3-GPTMS was successful.

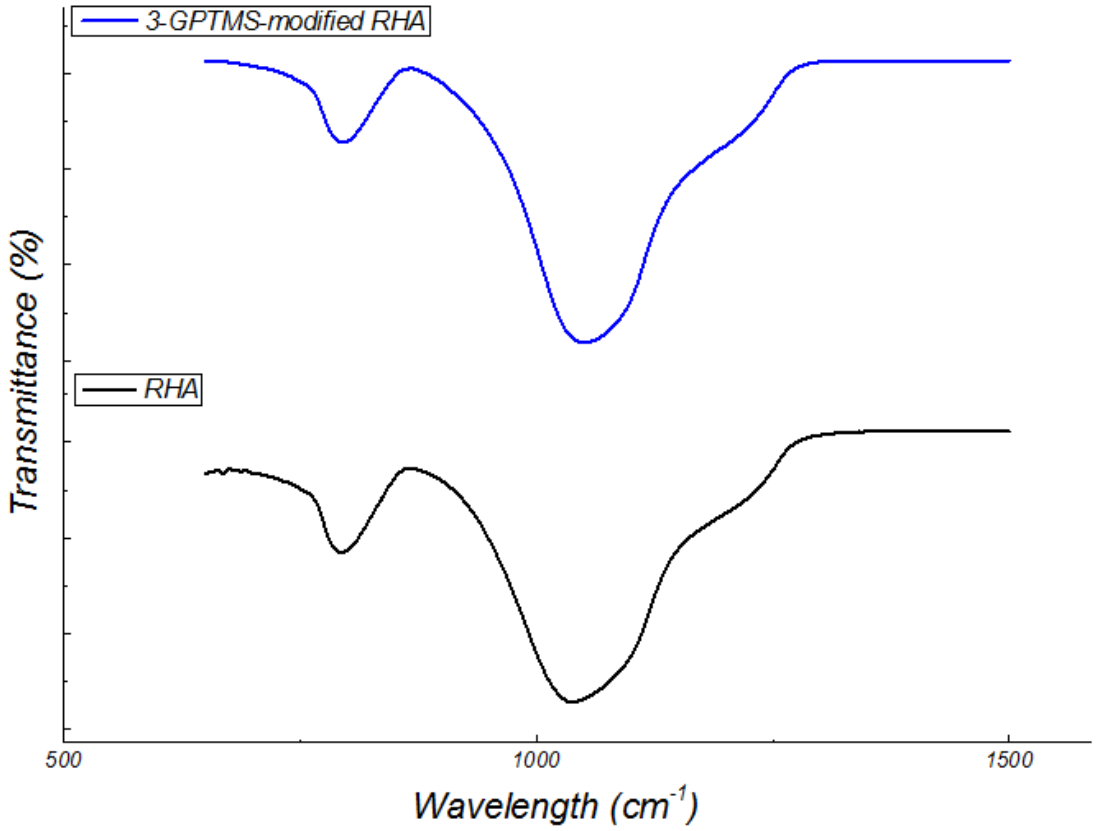

Figure 4.1: FT-IR analysis of 3-GPTMS-modified RHA.

In the region, in which the FT-IR analysis is given, there is also another peak at around 1050 cm^{-1}. This peak is due to Si-O-Si asymmetric stretching which is characteristic for silica based materials [57].

In Figure 4.2, FT-IR analysis of 3-APTMS-modified RHA gives a weak peak at around 3200 cm^{-1}. This peak is a result of asymmetric stretching vibration of primary amines [57]. Such peak cannot be observed in the FTIR spectrum of the pure RHA. Since 3-APTMS has a primary amine structure as functional group on its surface, this may be interpreted as the silanization of RHA with 3-APTMS was successfully achieved.

Figure 4.2: FT-IR analysis of 3-APTMS-modified RHA.

Surfaced-modified RHA samples were also thermally characterized by TGA thermogram in order to observe the presence of silane coupling agents. TGA thermogram of 3-GPTMS-modified RHA was given in Figure 4.3. As can be seen in the figure, the cumulative weight loss of the 3-GPTMS-modified RHA sample is found to be 5.47 % temperature up to 650 °C by TGA thermogram. The cumulative weight loss of pure RHA up to this temperature was given as 1.7531 % in a previous study [58]. When compared to the TGA results of the pure RHA, it was observed that the difference between the two values, 3.7169 %, gives the amount of 3-GPTMS that was evaporated.

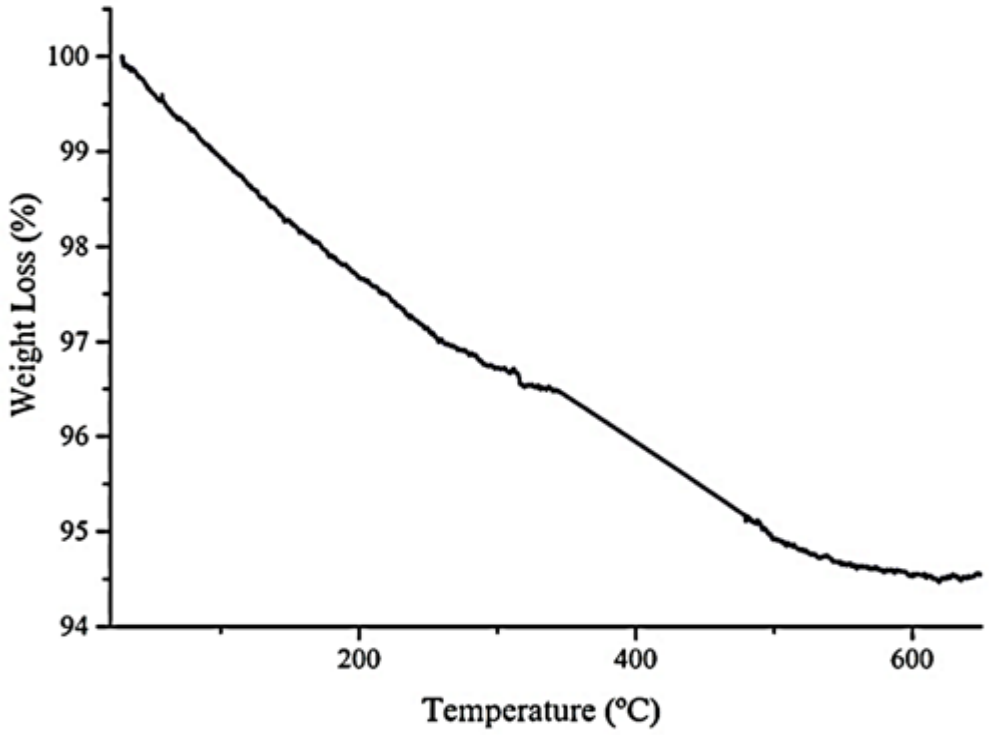

Figure 4.3: TGA analysis of 3-GPTMS-modified RHA.

Same calculation can be made for 3-APTMS-modified RHA sample. It can be seen in Figure 4.4 that the cumulative weight loss of 3-APTMS-modified sample is 2.528 %. The evaporated amount of 3-APTMS was calculated as 0.7749 %.

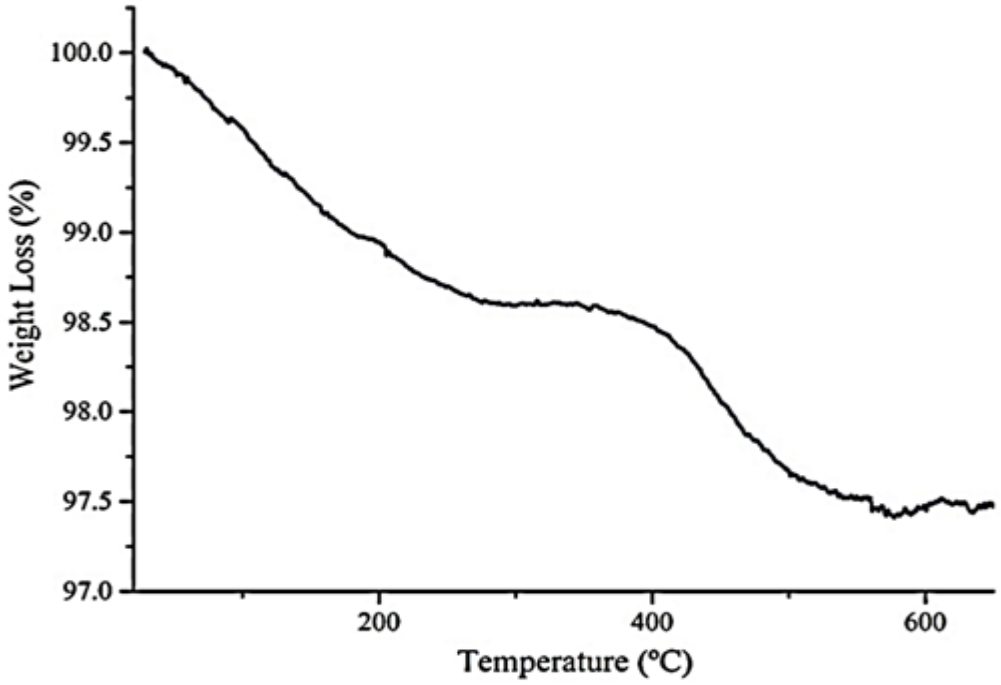

Figure 4.4: TGA analysis of 3-APTMS-modified RHA.

Surface morphologies of RHA and modified RHA samples were monitorized by SEM. It can be seen in Figure 4.5, RHA has a reticular surface texture with varying pore sizes. SEM images revealed that after silanization surface structure became smoother for both silanization agents. This might be due to the successful surface modification of RHA.

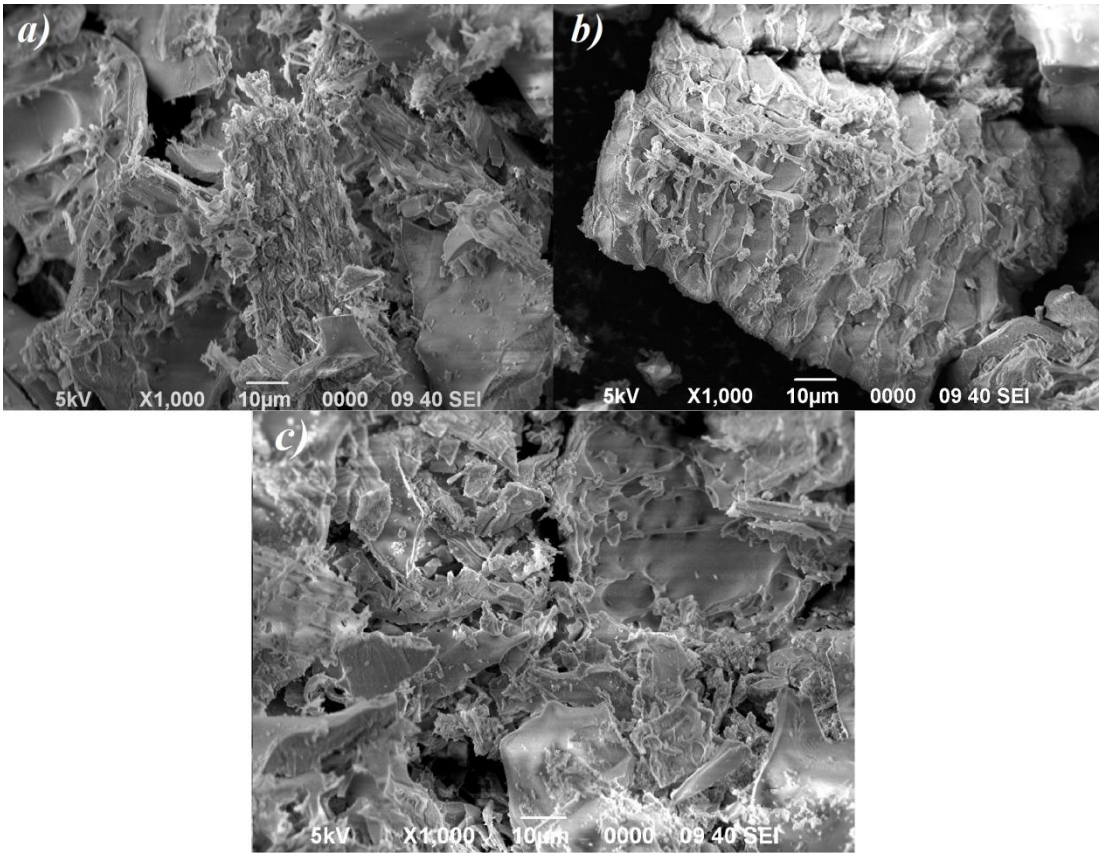

Figure 4.5: SEM images of a) RHA b) 3-GPTMS-modified RHA c) 3-APTMS-modified RHA.

As shown in Table 4.1, the surface area (S_{BET}) of unmodified RHA is found to be 50.61 m^2/g. It is known that surface area of RHA decreases with increasing combustion temperature during preparation [59]. The surface area decreases to 31.88 m^2/g when the silica is modified with 3-APTMS. This can be considered as a slight decrease and may be due to deposition of small species formed by hydrolysis reactions [60]. On the other hand, cumulative pore volume is significantly increased. In case of 3-GPTMS modification, surface area increases to 90.28 m^2/g, which may be attributed to the formation of new pore structures by 3-GPTMS-silica complexes [61]. Both 3-GPTMS and 3-APTMS-modified RHA have suitable pore sizes for CALB immobilization whose particle size is given as 6.9 nm x 5.05 nm x 8.67 nm [22].

Table 4.1: Surface areas and pore sizes of the RHA samples.

	RHA	3-GPTMS-modified RHA	3-APTMS-modified RHA
BET Surface Area (S_{BET}) (m^2/g)	50.61	90.28	31.88
BJH Adsorption Cumulative Pore Volume (pores 1.7-300 nm) (cm^3/g)	0.196	0.251	0.156
BJH Adsorption Average Pore Diameter (nm)	14.26	9.60	21.46

4.2 Immobilization of Lipase

Lipase enzyme was immobilized onto 3-GPTMS-modified and 3-APTMS-modified RHA by physical adsorption method. FTIR analysis of all immobilization steps for both 3-GPTMS and 3-APTMS modifications be seen in Figure 4.6 and Figure 4.7, respectively.

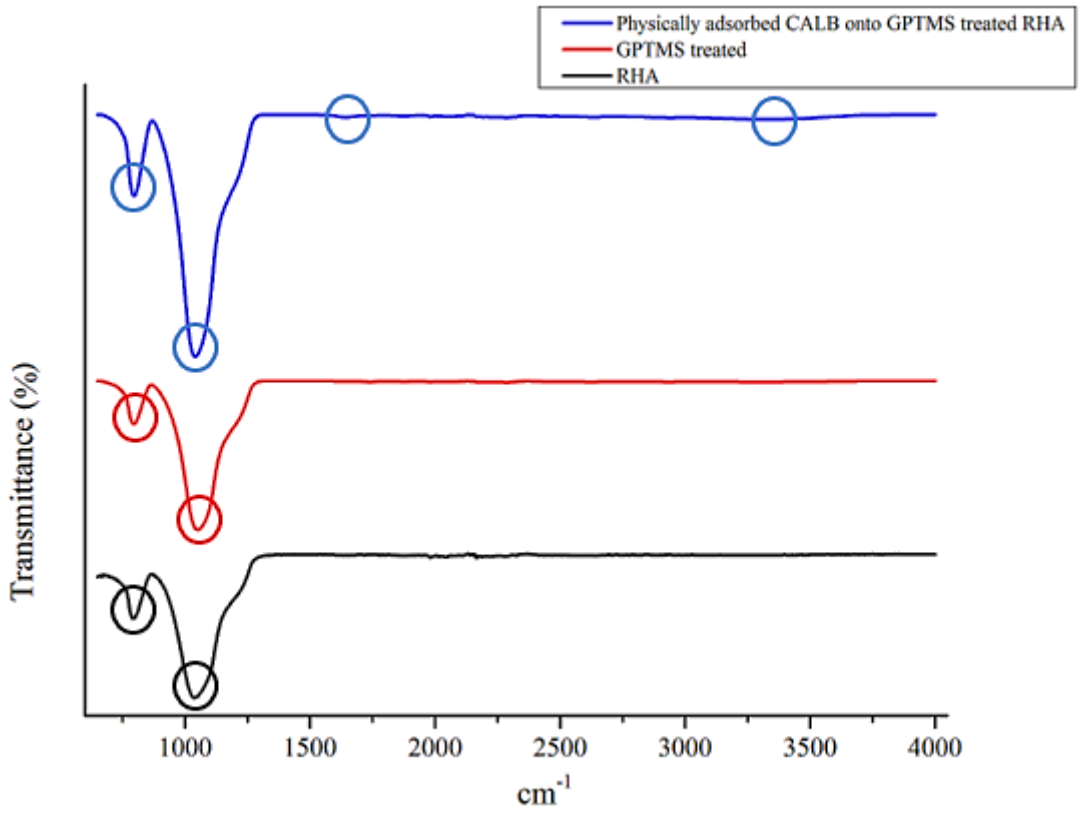

Figure 4.6: FT-IR spectra of immobilization steps with 3-GPTMS modification.

There are two observable peaks present in all of the given spectra, RHA, 3-GPTMS-modified RHA, 3-APTMS-modified RHA, immobilized lipase onto 3-GPTMS-modified RHA and immobilized lipase onto 3-APTMS-modified RHA. First one is at about 800 cm^{-1} and it represents Si-CH_3 rocking [57]. Second one is a characteristic peak for silica based materials and at around 1050 cm^{-1}. It is caused by asymmetric stretching vibrations of Si-O-Si bond structure [57, 62]. At 1650 cm^{-1}, a peak with low intensity is observed in the spectra of immobilized lipase. This peak may be caused by C=O stretch and NH_2 deformation [57, 63]. Additionally, there is another peak that is observed only in enzyme spectra. This peak is between 3250-3300 cm^{-1} and may be due to -OH stretching present in carboxylic acid [57]. These two peaks show that lipase immobilization onto 3-GPTMS-modified and 3-APTMS-modified RHA is successful. There is also a slight peak, which is not very observable, at about 2900 cm^{-1} which due to –CH_3 absorbance [27]. It can only be caused by methoxy groups of 3-GPTMS and 3-APTMS thus it can be concluded that there may be small amount of unreacted 3-GPTMS and 3-APTMS.

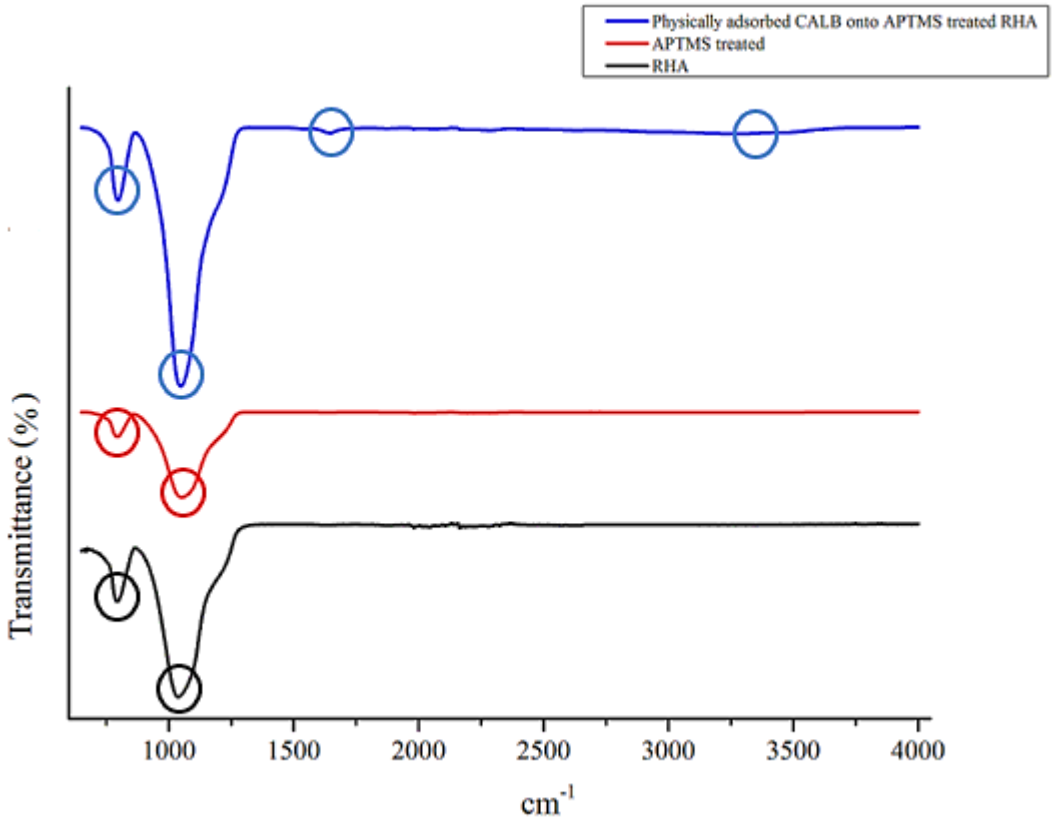

Figure 4.7: FT-IR spectra of immobilization steps with 3-APTMS modification.

The results of immobilization experiments are tabulated in Table 4.2. Lipase immobilization is successfully completed for both silanization agents, with an immobilization efficiency of approximately 90 %. Additionally, almost 80 % of catalytic activity of lipase enzyme was remained after immobilization for both 3-GPTMS and 3-APTMS modification. Although 80 % is an acceptable value, it is reported that 92 % of catalytic activity is remained with 3-APTES modification.

Table 4.2: Immobilization of CALB onto RHA modified with different silanization agents.

Silanization Agent	Immobilization Efficiency (%)	Relative Activity (%)
3-GPTMS	88	78
3-APTMS	91	77
3-APTES*	90.7	92.3

* Results were obtained from [55].

Specific activities of the immobilized lipases were compared with Novozyme 435® and free lipase (Lipozyme®) which can be seen in Figure 4.8. Specific activities of both 3-GPTMS and 3-APTMS modified samples were close to that of Novozyme 435® (10 U/mg) [54]. In addition, specific activity values were improved by 1.8 and 1.7 folds for 3-GPTMS and 3-APTMS modification respectively, compared to free lipase.

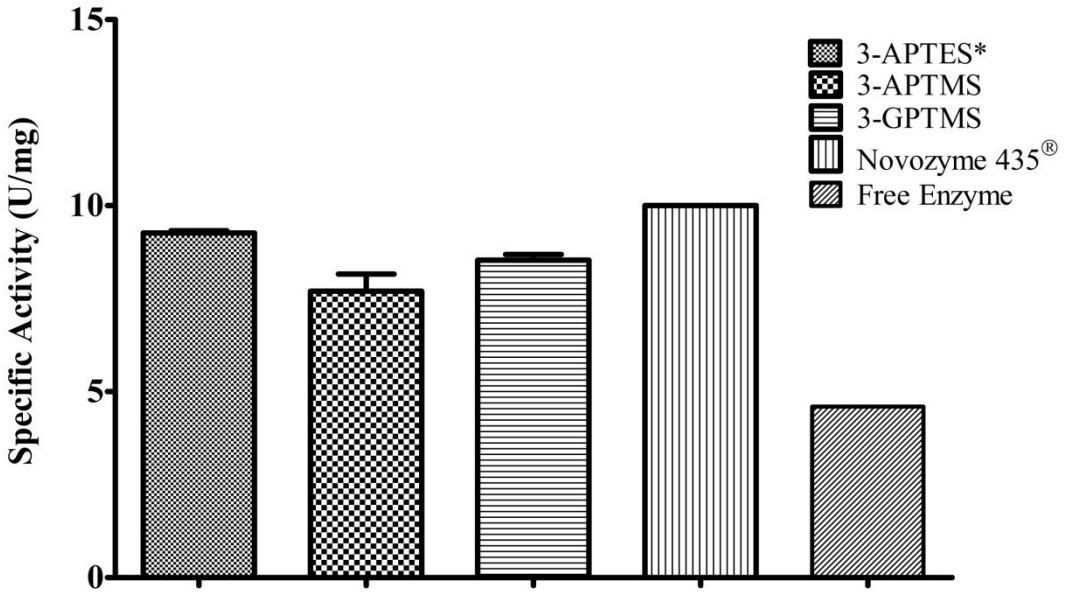

Figure 4.8: Specific activities of immobilized CALB onto RHA modified with different silanization agents. (*Results were obtained from [55]).

Surface morphologies of immobilized lipases were also observed with SEM imaging which can be seen in Figure 4.9.

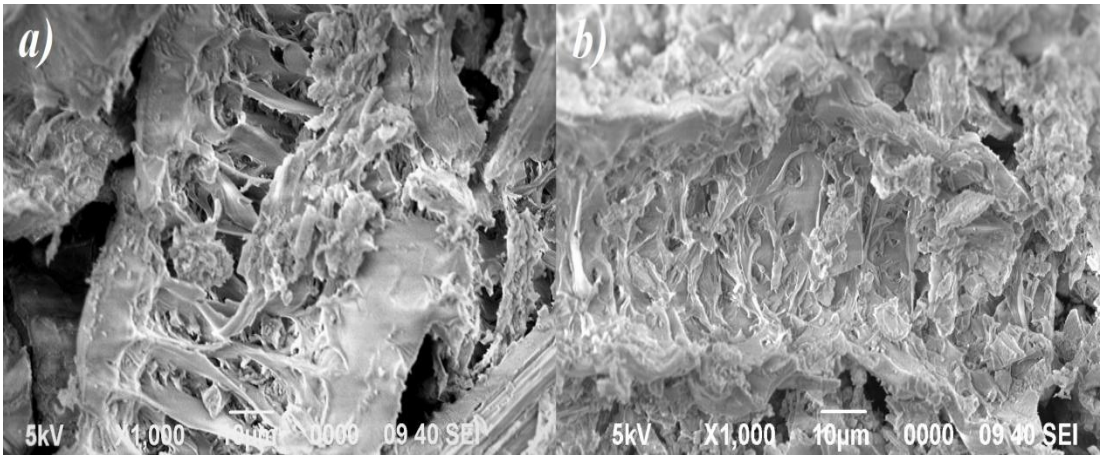

Figure 4.9: SEM images of lipase immobilized onto a) 3-GPTMS-modified RHA b) 3-APTMS-modified RHA.

The main characteristic surface structure of immobilized enzyme onto 3-GPTMS-modified RHA is the bond structure, which can be interpreted as the immobilization of lipase onto 3-GPTMS-modified RHA (epoxy-activated material, in general) is achieved through covalent bonding between the support and the enzyme. On the other hand, such bond structures cannot be observed for lipase enzyme immobilized onto 3-APTMS-modified RHA.

4.3 Storage Stability

One of the major concerns in biocatalysis is the shelf life of the enzymes. For this reason, storage stabilities of the immobilized lipase samples were determined. The relative activity changes of the samples in 3 month period were investigated and are given in Figure 4.10. For enzyme samples immobilized onto RHA modified with both types of silanization agents, relative activities were dropped to approximately 45 % after 3 months of storage. It can be seen in Figure 4.10 that immobilized CALB onto 3-APTMS-modified RHA shows a steep decrease after a month of storage at 4 °C. On the other hand, the relative activity of the immobilized CALB onto 3-GPTMS-modified RHA decreased rapidly after 15 days of storage at 4 °C and later the decrease slowed down.

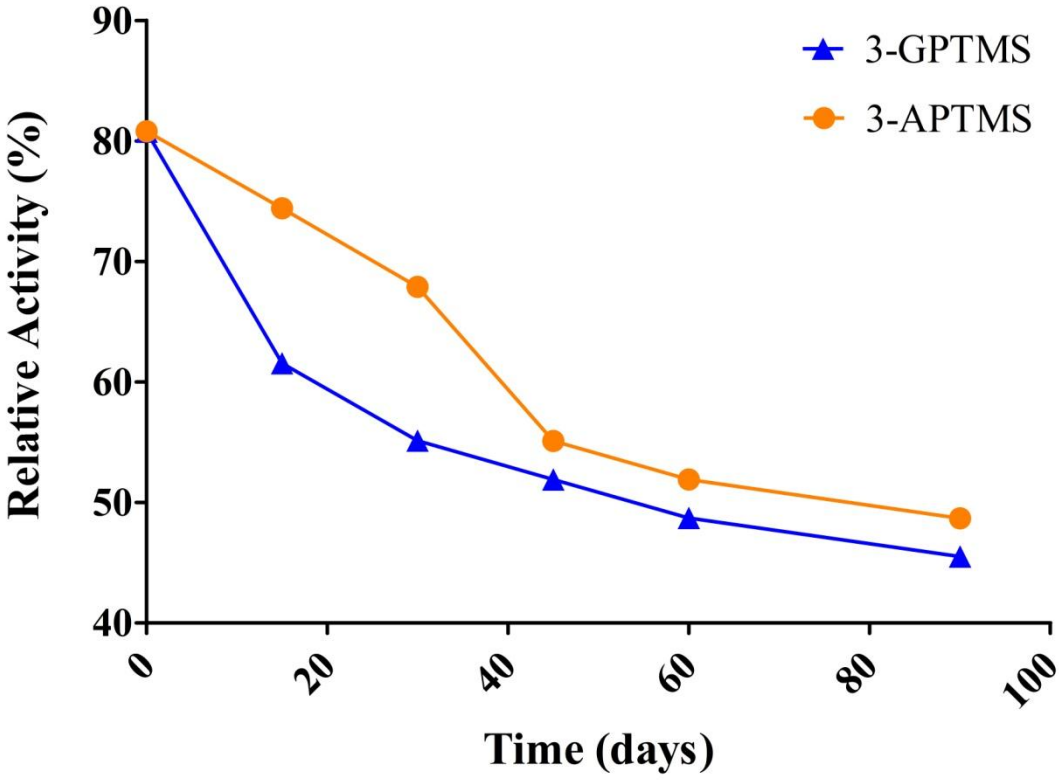

Figure 4.10: Storage stability of immobilized CALB samples.

4.4 Factors Affecting Catalytic Activity of Immobilized CALB

4.4.1. Effect of temperature

The effect of temperature is a critical factor that affects the catalytic activity of the immobilized enyzme. For this reason both optimum temperature and temperature stability of immobilized CALB onto 3-GPTMS and 3-APTMS-modified RHA samples were determined.

Optimum temperatures of the immobilized CALB samples were determined by measuring activities after operating the enzyme samples at different temperatures. The temperature of the optimum temperature experiments ranged from 30 °C to 60 °C. The change of relative activities (ratio of activity of the sample to the activity of the sample with the highest value) were measured by applying the same activivty measurement procedure explained in the earlier sections and is given in Figure 4.11. The change of relative activities of the immobilized CALB samples were also compared to that of Novozyme 435® and free CALB.

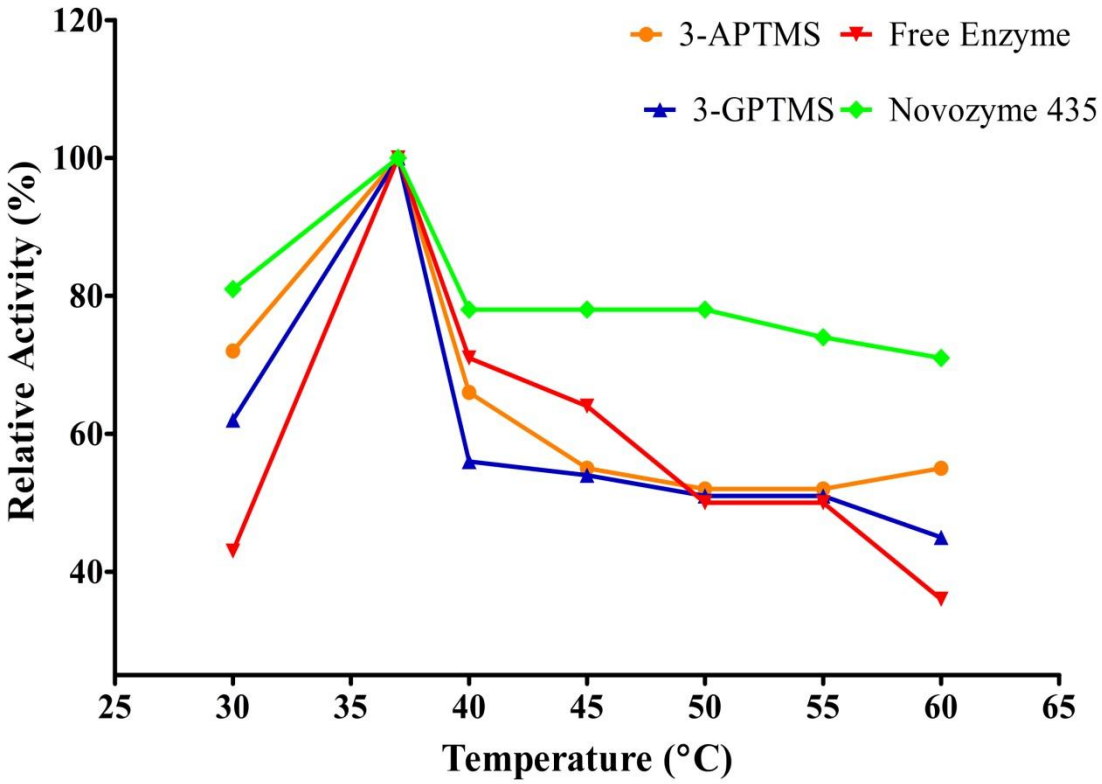

Figure 4.11: Optimum temperature of immobilized CALB samples.

As it can be clearly seen in the figure, the optimum operating temperature of both immobilized enzyme samples is found to be 37 °C. This value is also the optimum temperature for Novozyme 435® and free lipase. At higher temperatures, activity of immobilized CALB onto 3-GPTMS and 3-APTMS-modified RHA decreases rapidly, dropping around 50 % of the highest value at 37 °C. On the other hand, Novozyme 435® retained almost 80 % of its highest activity value at 37 °C, at higher temperatures.

Temperature stability curves of the immobilized enzyme samples are given in Figure 4.12. Thermal stabilities of the immobilized samples were also compared with free eznyme and Novozyme 435® Both CALB immobilized on 3-GPTMS-modified and 3-APTMS-modifed RHA are thermally stable at a narrower interval with respect to free lipase and Novozyme 435®. Both immobilized CALB samples seem to be thermally stable between 0 – 50 °C. It can be said for free lipase and Novozyme 435® that they are thermally stable even at very high temperatures like 70-80 °C.

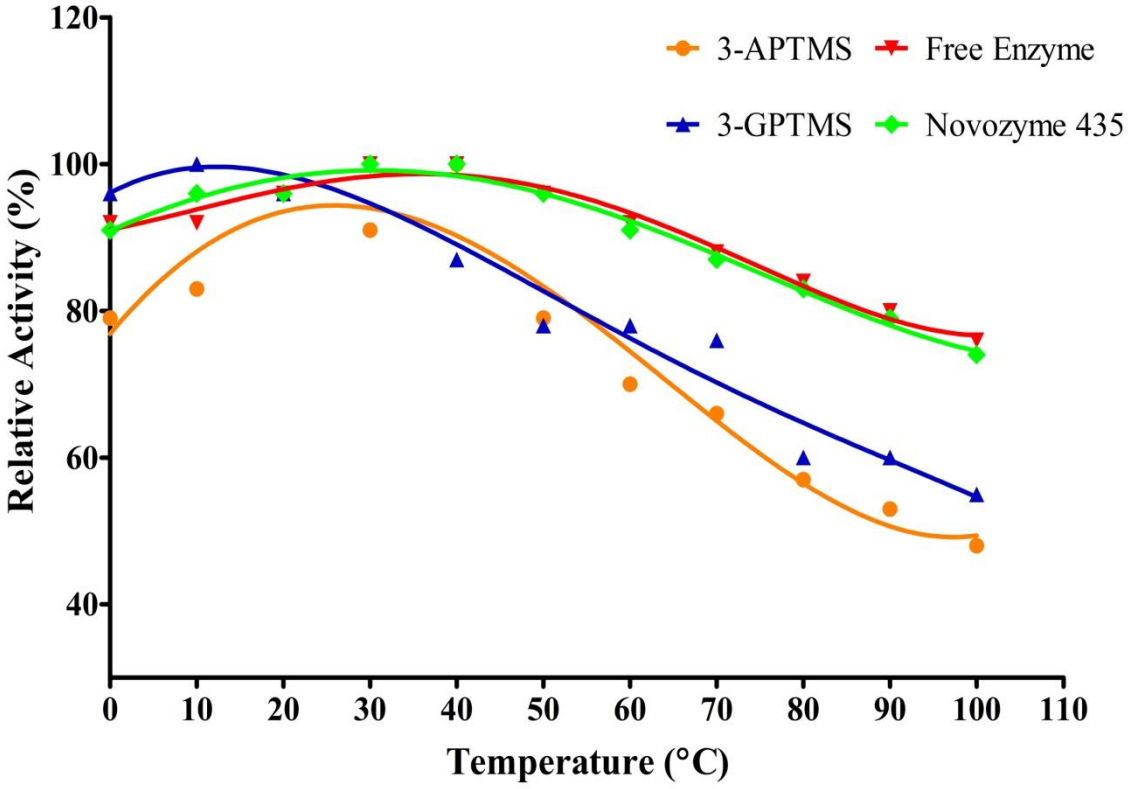

Figure 4.12: Temperature stability of immobilized CALB samples.

4.4.2. Effect of pH

pH is another important parameter that affects the performance of the immobilized enyzme. For this reason both optimum pH and pH stability of immobilized CALB onto 3-GPTMS and 3-APTMS-modified RHA samples were determined.

Optimum pH of immobilized CALB were determined by measuring the relative activities at different pH values and given in Figure 4.13. As can be seen in the figure, both immobilized CALB samples have optimum relative activity value at pH 7.0. At basic region, 3-APTMS modification seems to be slightly more efficient than 3-GPTMS modification. It is also obvious that both CALB immobilized onto 3-GPTMS and 3-APTMS-modified RHA have higher activity than free enzyme at both acidic and basic regions. At basic regions immobilized CALB samples seem to be more active than Novozyme 435®, while at acidic regions Novozyme 435® is more active. Thus, it can be concluded that these immobilized lipases can be an alternative to Novozyme 435® for catalysis in basic reaction mediums.

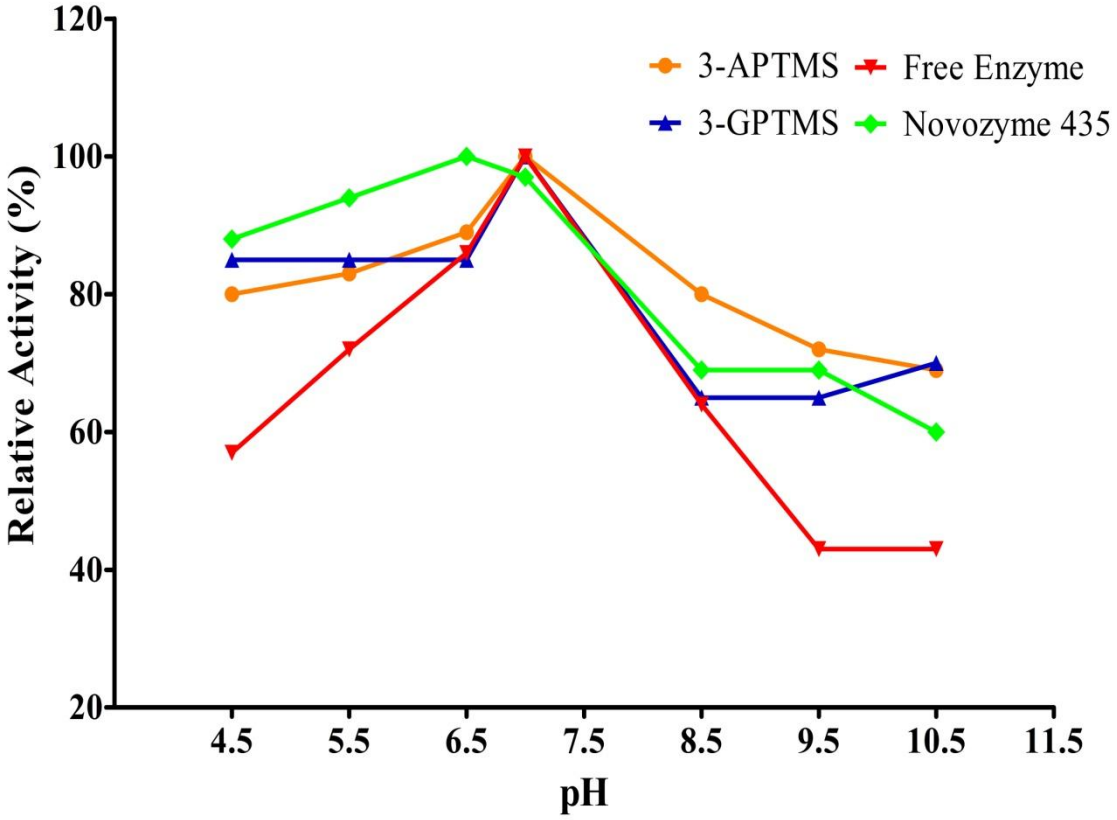

Figure 4.13: Optimum pH of immobilized CALB samples.

Stability curves of the CALB samples immobilized onto 3-GPTMS and 3-APTMS-modified RHA, at a range of pH 4.5 – 10.5 are given in Figure 4.14. The change of relative activities between this pH range were also compared to those of free enzyme and Novozyme 435®. The trend of the stability curve of CALB immobilized onto 3-APTMS-modified RHA is similar to that of Novozyme 435®. They are stable between pH 4.5 – 8.5; this region also contains the optimum pH values which is pH 7.0. CALB immobilized onto 3-GPTMS-modified RHA has a narrower range of pH stability with respect to CALB immobilized onto 3-APTMS-modified RHA, between pH 4.5 – 7.5. The optimum pH value of immobilized CALB onto 3-GPTMS-modified RHA sample, pH 7.0, is also in the stable range of this immobilized enzyme sample. Both immobilized CALB onto 3-GPTMS and 3-APTMS- modified RHA seem to be stable at weak acidic or neutral media rather than extreme conditions such as strong acidic or basic conditions.

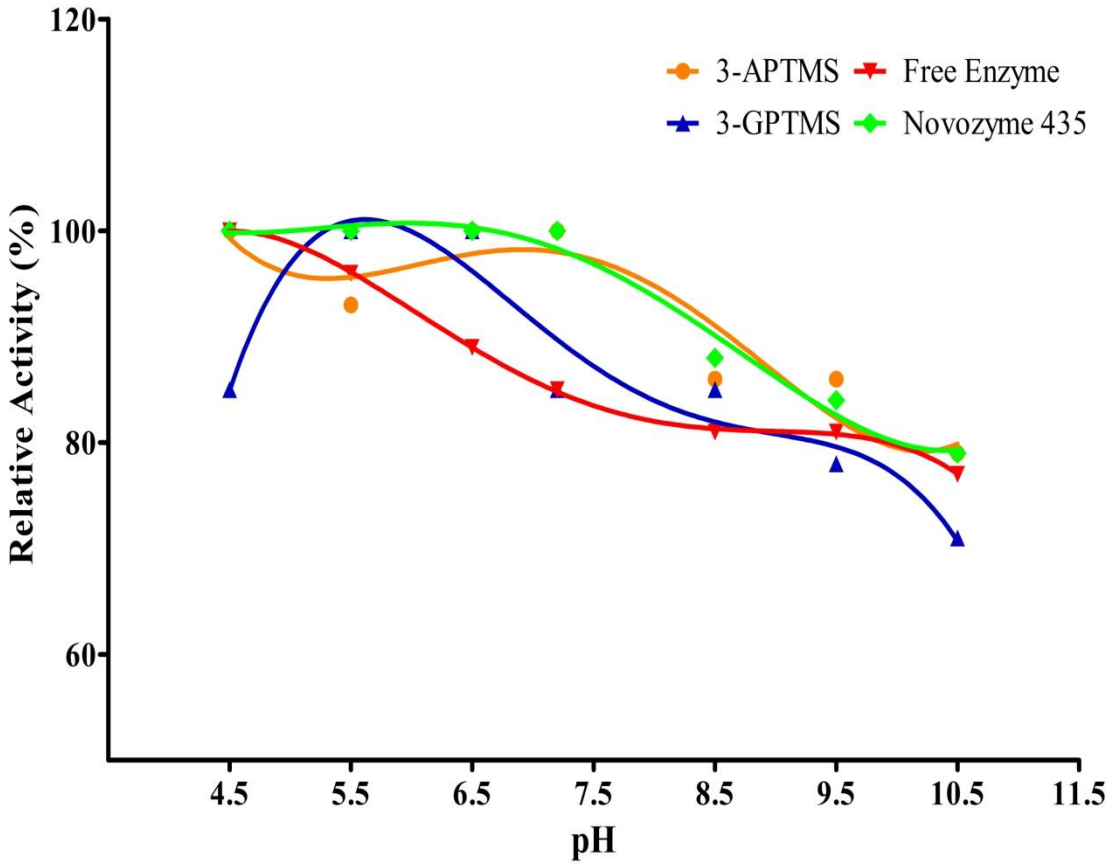

Figure 4.14: pH stability of immobilized CALB samples.

4.4.3. Effect of silane concentration

One of the parameters that affect the catalytic activity of the immobilized enzymes is the silanization agent concentration. As a general consideration, it is not preferred to consume excess amounts of raw material due to economic reasons. Thus, high silanization agent concentrations are not advantageous. The effect of silanization agent concentration on the catalytic activity of immobilized CALB onto 3-GPTMS and APTMS-modified RHA is given in Figure 4.15. High silanization agent concentration (30 %) obviously has an adverse effect on catalytic activity for both 3-GPTMS and 3-APTMS modification, decreasing relative activity to approximately 50 %. This may be due to the interactions between excess silane and the active sites of the lipase enzyme. In addition, 5 % silane concentration does not seem to be efficient. For CALB immobilized onto 3-GPTMS-modified RHA, highest relative activity value was reached with 15 % v/v silane concentration, 80 % for both 20 % and 30 % enzyme loading.

For CALB samples immobilized onto 3-APTMS-modified RHA, there are three data points that have remarkable relative activity value; 20 % silane concentration – 5 % enzyme loading, 15 % silane concentration – 30 % enzyme loading and 10 % silane concentration – 5 % enzyme loading.

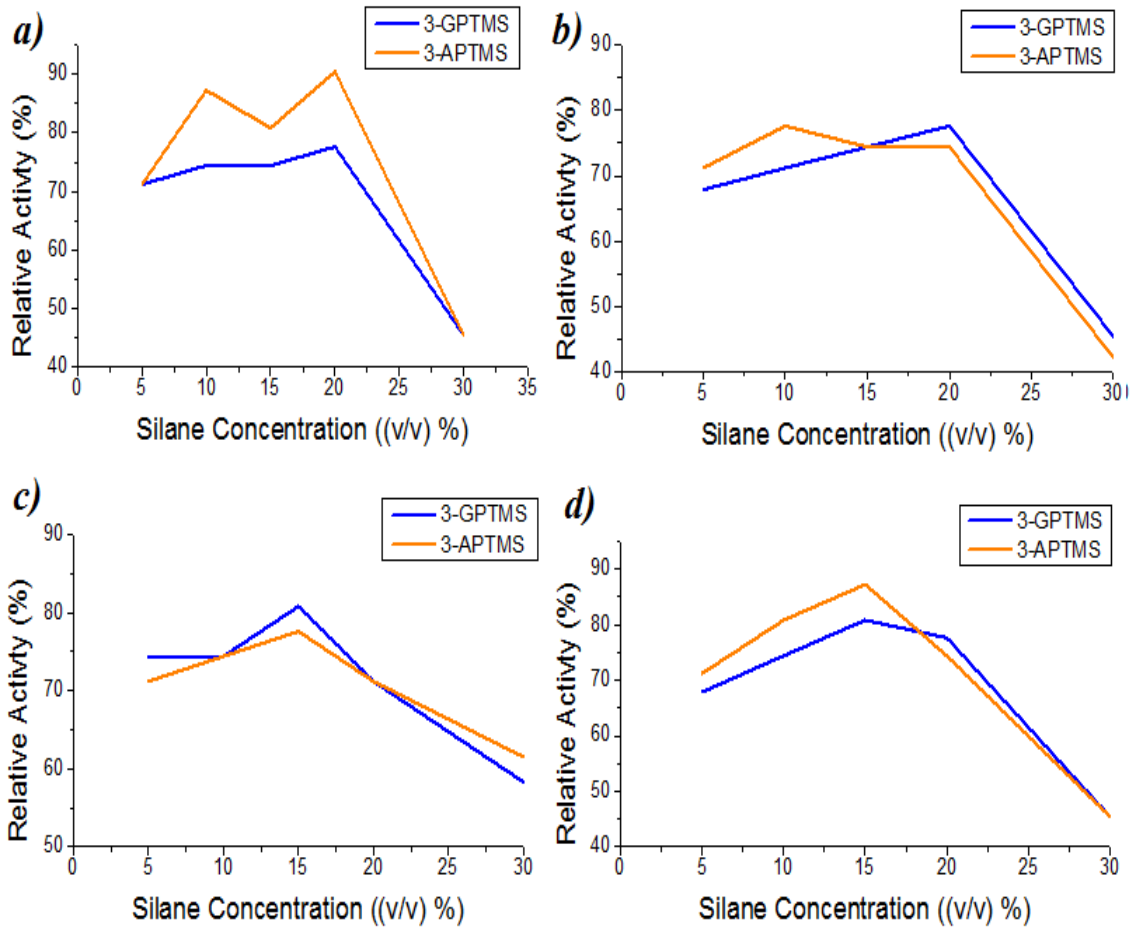

Figure 4.15: Effect of silanization agent concentration on catalytic activity, a) 5 %, b) 10 %, c) 20 %, d) 30 % enzyme loading.

In order to observe the effect of silanization agent concentration on catalytic activity, different silanization agents were used with specific ratios during silanization procedure. For this purpose, 1:1 (v/v) 3-APTMS/3-GPTMS and 3-APTES/3-GPTMS mixtures are prepared and same amount (15 % (v/v)) of these new silanization agent mixtures were used during the silanization process. Immobilization was applied with the same procedure explained earlier. The results are summarized in Table 4.3. As can be seen in the table, specific activities were decreased almost by 50 % in case of silanization with silane mixtures. While CALB immobilized onto 3-APTES-modified RHA has a very high specific activity (9.3 U/mg), the activity decreases to 5.5 U/mg

when silanization was applied with a mixture with 3-GPTMS. Similarly, CALB immobilized onto 3-GPTMS and 3-APTMS-modified RHA have specific activities around 8 U/mg, but the specific activity is reduced to 5.6 U/mg in case of mixture of these two silanization agents. This may be attributed to different functionalization of the support material due to different types of silanization agents. Similar results can be found in literature, also [10]. Thus, it can be concluded that monofunctional support materials, rather than heterofunctional support materials, yield more active immobilized enzymes.

Table 4.3: Specific activities of enzymes prepared with different silanization agents.

Silanization Agent	Specific Activity (U/mg)
3-GPTMS	8.3
3-APTMS	7.7
3-APTES*	9.3
3-APTMS/3-GPTMS	5.6
3-APTES/3-GPTMS	5.5

* Results were obtained from [55].

4.4.4. Effect of enzyme loading

Enzyme loading ratio is an important parameter related to enzyme immobilization and catalytic activity of immobilized enzymes. For this reason, different enzyme loading ratios (5 %, 10 %, 20 % and 30 %) were tried in order to observe the effects of enzyme loading ratio on catalytic activity of immobilized CALB. The effect of enzyme loading ratio on catalytic activity of immobilized CALB onto 3-GPTMS-modified RHA is given in Figure 4.16. Relative activities range from around 45 % to 80 %, as seen in the figure. In general, 20 % enzyme loading yields high catalytic activity. Highest catalytic activity, around 80 % relative activity, is reached with 15 % silane concentration and 20 % enzyme loading ratio. Additionally, there are few data points which yields catalytic activity close to the highest value, reaching around 78 %. All of these data points are prepared with 20 % silane coupling agent concentration and the enzyme loading ratios are 5 %, 10 % and 30 %. Also, except of 20 % silane concentration, 5 % and 10 % enzyme loading ratio seems to yield low catalytic activity compared to the other ratios (20 % and 30 %).

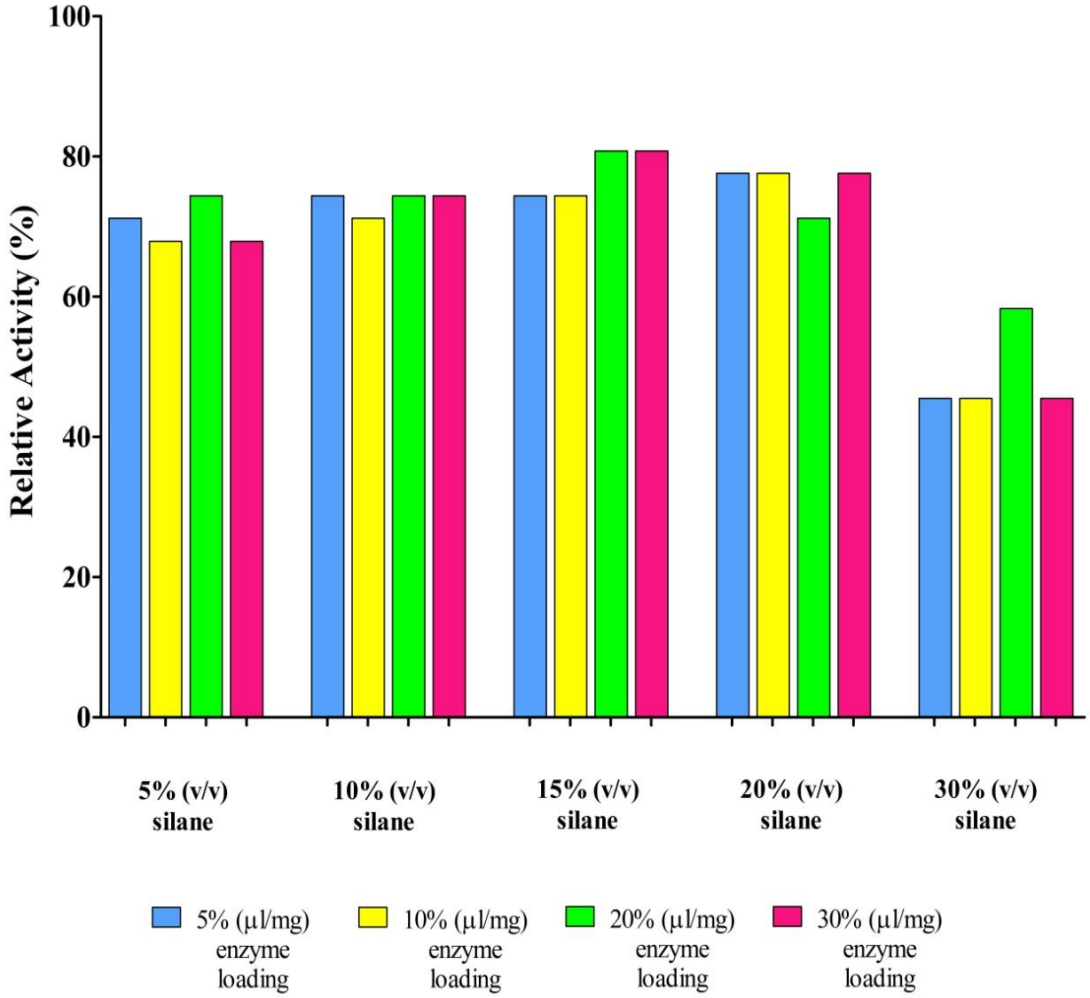

Figure 4.16: The effect of enzyme loading on the catalytic activity of immobilized CALB onto 3-GPTMS-modified RHA.

In Figure 4.17, the effect of enzyme loading ratio on the catalytic activity of immobilized CALB onto 3-APTMS-modified RHA is given. When 5 % silanization agent concentration is used, it seems that there is no effect of enzyme loading ratio on the catalytic activity. Relative activities remain constant at around 70 %. For 10 %, 15 % and 20 % silanization agent concentration, 10 % and 20 % enzyme loading yields lower catalytic activity, around 70 % relative activity. In addition, compared to 3-GPTMS modification, 3-APTMS modification required less enzyme loading in order to reach its highest catalytic activity, with 5 % enzyme loading. This may be due to undesired covalent attachments between the epoxy rings of the 3-GPTMS-modified RHA and the active sites of the enzyme. However, 3-APTMS modification also requires more silanization agent concentration with respect to 3-GPTMS modification.

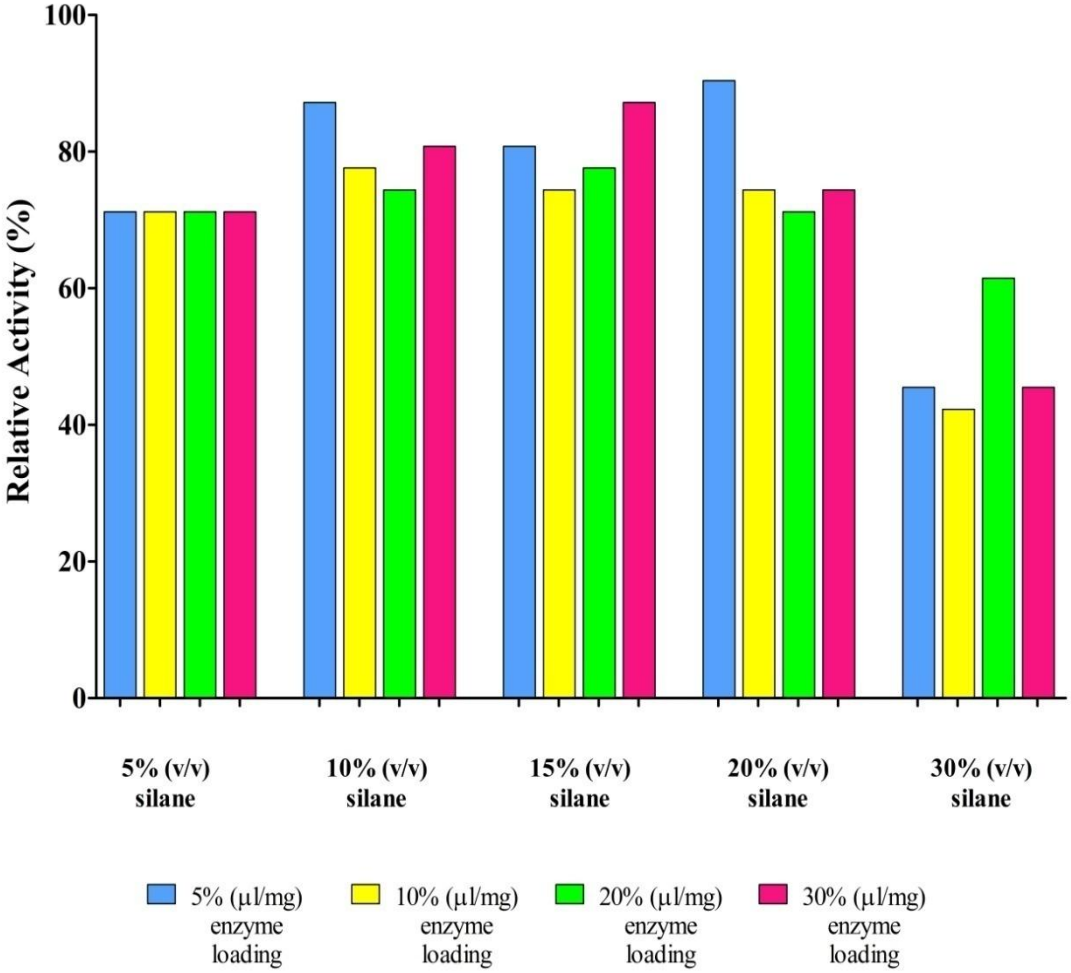

Figure 4.17: The effect of enzyme loading on the catalytic activity of immobilized CALB onto 3-APTMS-modified RHA.

4.5 Effect of Silane Concentration and Enzyme Loading on Immobilization Efficiency of Immobilized CALB

Immobilization efficiencies of immobilized CALB samples with different silanization agent concentration and enzyme loading ratio are given in Table 4.4. The efficiencies are rather similar to each other; in other words, immobilization efficiency is not affected significantly by the change of silanization agent concentration and enzyme loading ratio. However, for economic reasons, it is not preferred to consume excess amounts of raw material. Thus, high silanization agent concentrations and high enzyme loading ratios are not advantageous. For immobilized CALB onto 3-GPTMS-modified RHA, there are several data points with immobilization efficiency of 90 % or more, such as 5 % silanization agent concentration – 10 % enzyme loading, 10 % silanization agent concentration – 30 %

enzyme loading, 20 % silanization agent concentration – 10 % enzyme loading and 30 % silanization agent concentration – 10 % enzyme loading. 30 % silanization agent concentration or enzyme loading is not preferred due to the reasons that are explained. The immobilization efficiencies of the CALB samples immobilized onto 3-APTMS-modified RHA are generally higher than those of CALB samples immobilized onto 3-GPTMS-modified RHA. It seems that samples with 5 % silanization agent concentration yield better immobilization efficiencies, about 90 %. Also, high silanization agent concentrations yield high efficiencies though they are not feasible since high consumption of raw materials.

Table 4.4: Effects of silanization agent concentration and enzyme loading ratio on immobilization efficiency.

Silanization Agent Concentration (% v/v)	**Enzyme Loading Ratio (µL/mg)**	**Immobilization Efficiency (%)**	
		3-GPTMS	3-APTMS
5	5	86	90
	10	92	93
	20	88	90
	30	88	92
10	5	82	87
	10	81	89
	20	87	91
	30	91	89
15	5	82	82
	10	84	87
	20	88	91
	30	83	91
20	5	86	89
	10	90	89
	20	87	93
	30	89	90
30	5	86	92
	10	91	93
	20	86	89
	30	86	91

By considering immobilization efficiencies and catalytic activivties, it can be safely concluded that 15 % silanization agent concentration and 20 % enzyme loading is the optimum condition for CALB immobilization onto 3-GPTMS-modified RHA since its immobilization is slightly higher. For immobilized CALB onto APTMS-modified RHA, there are three alternatives to the ratios used in this study. When compared to 15 % silane concentration – 20 % enzyme loading, both 20 % silane concentration –

5 % enzyme loading and 15 % silane concentration – 30 % enzyme loading have lower immobilization efficiency and use more silane and enzyme. However, 10 % silane concentration – 5 % enzyme loading might be an alternative in terms of immobilization efficiency and catalytic activity.

Mixtures of different silanization agents were also investigated in terms of their effect on immobilization efficiencies. As given in Figure 4.18, the results were found to be very close to each other, which can be interpreted as mixtures of different silanization agents yield approximately the same immobilization efficiencies as the pure silanization agents.

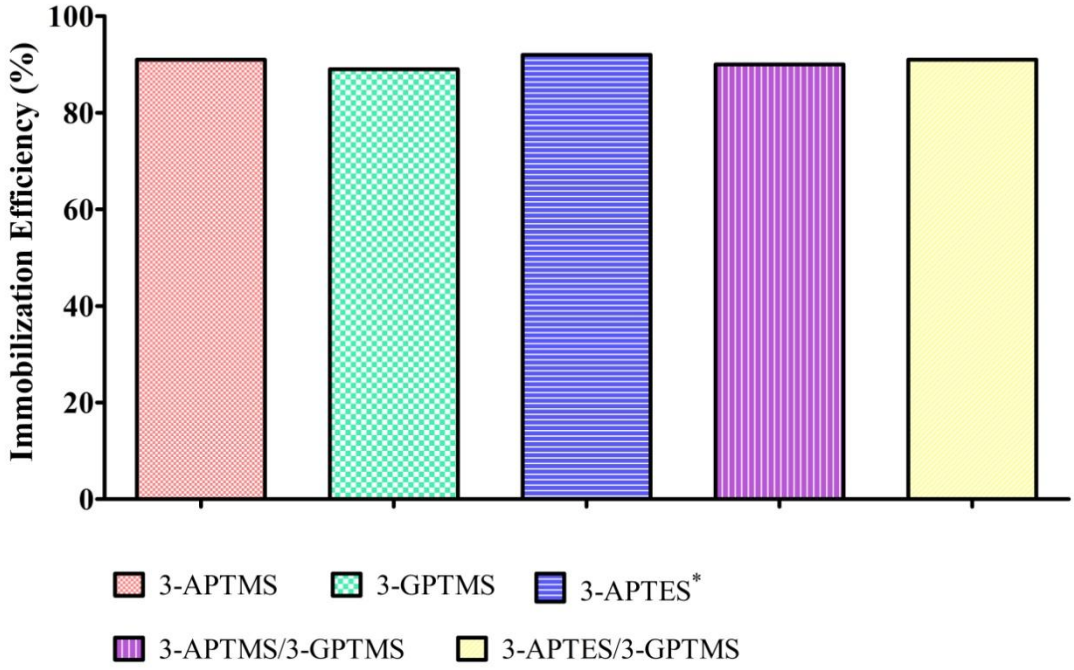

Figure 4.18: Immobilization efficiencies of enzymes prepared with different silanes.

4.6 Operational stability

Reusability of the immobilized CALB samples onto 3-GPTMS and 3-APTMS-modified RHA are given in Figure 4.19. Reusability is an important property of enzymes for industrial applications since it allows use of enzymes in continous processes. Both immobilized CALB samples retained approximately 50 % of their initial activity after 14-repeated use.

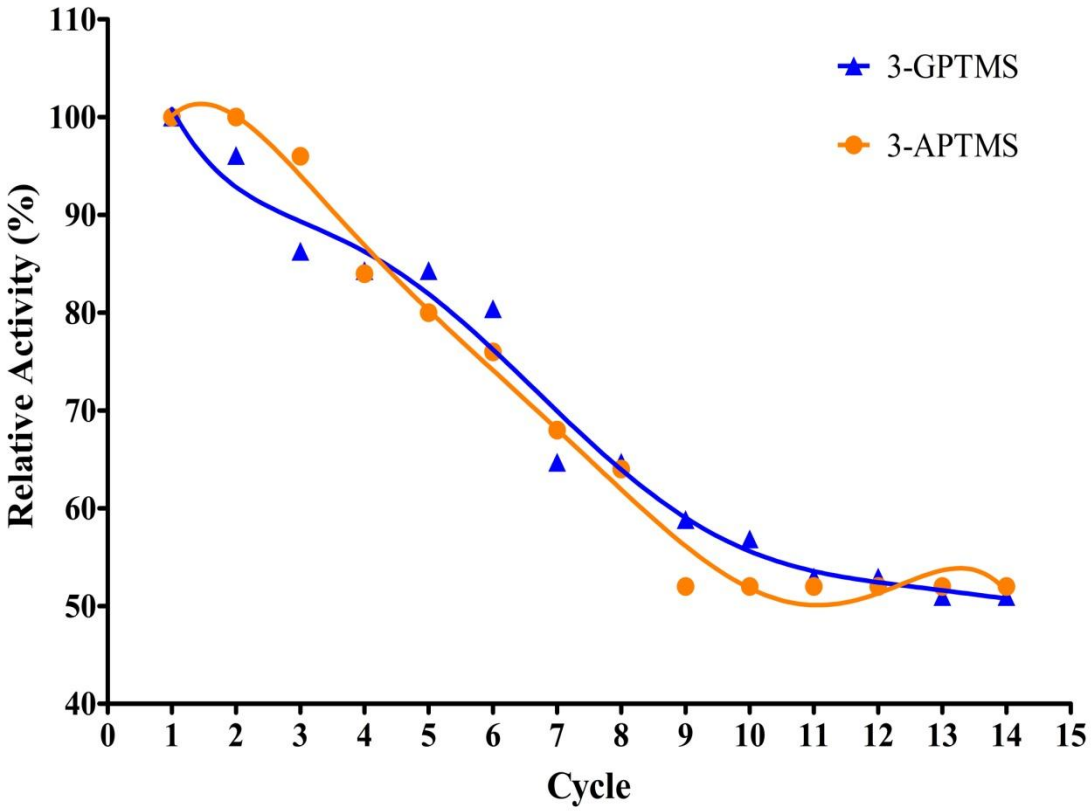

Figure 4.19: Operational stability of immobilized CALB samples.

5. CONCLUSIONS AND RECOMMENDATIONS

In this study, CALB immobilization onto surface-modified RHA by physical adsorption method is investigated. For surface modification of RHA, two different silanization agents were used, 3-GPTMS and 3-APTMS. In case of 3-GPTMS modification, RHA was modified through the epoxy group whereas 3-APTMS modification was achieved through $–NH_2$ group of 3-APTMS. After surface modification, free CALB was immobilized by physical adsorption method. Some specific properties of these immobilized enzymes were determined in order to decide whether these novel catalysts are promising or not. Immobilization efficiencies of the immobilized CALB samples were determined by UV spectrophotometer. Catalytic activities of the immobilized enzyme samples were determined by titrimetric method. Additionally, the effects of pH and temperature on the catalytic activity of the immobilized enzyme samples were investigated. Storage and operational stability experiment were conducted in order to determine shelf life and reusability of the immobilized enzyme samples. The effect of some parameters (silanization agent concentration, enzyme loading ratio) on immobilization efficiency and catalytic activity of the immobilized CALB samples were studied. Furthermore, the surface-modified RHA and the immobilized CALB samples were characterized by FT-IR and TGA. Surface morphologies of modified RHA and immobilized CALB samples were monitorized by SEM.

Firstly, modified RHA was characterized by using FT-IR and TGA analysis in order to observe the presence of the functional groups (epoxy and $–NH_2$) obtained on the surface of RHA after silanization. In FT-IR spectrum, there exists a peak at 800 cm^{-1} which corresponds to epoxy ring of 3-GPTMS [27]. This is also a peak for $Si-CH_3$ rocking [57]. In TGA analysis, 3.7169 % weight loss is found to belong to 3-GPTMS. In the FT-IR spectrum of the 3-APTMS-modified RHA, a slight peak is observed at 3200 cm^{-1} which may be the result of asymmetric stretching vibration of primary amines [57]. Also in TGA analysis, a weight loss of 2.528 % which is 3-APTMS. According to these results, it is safely concluded that silanization with both

silanization agents is successfully completed. Surface morphologies of RHA and modified RHA samples were monitorized with SEM. SEM images revealed that after silanization surface structure became smoother for both silanization agents. This might be due to the successful surface modification of RHA. After characterization of modified RHA samples, immobilized enzymes were also characterized with FT-IR analysis. Two peaks are observed in all of the spectra of RHA, 3-GPTMS-modified RHA, 3-APTMS-modified RHA, immobilized lipase onto 3-GPTMS-modified RHA and immobilized lipase onto 3-APTMS-modified RHA. First one is at about 800 cm^{-1} and it represents Si-CH_3 rocking [57]. Second one is a characteristic peak for silica based materials and at around 1050 cm^{-1}. It is caused by asymmetric stretching vibrations of Si-O-Si bond structure [57, 62]. At 1650 cm^{-1}, a peak with low intensity is observed in the spectra of immobilized lipase. This peak may be caused by C=O stretch and NH_2 deformation [57, 63]. Additionally, there is another peak that is observed only in enzyme spectra. This peak is between 3250-3300 cm^{-1} and may be due to -OH stretching present in carboxylic acid [57]. These two peaks show that lipase immobilization onto 3-GPTMS-modified and 3-APTMS-modified RHA is successful. There is also a slight peak, which is not very observable, at about 2900 cm^{-1} which due to –CH_3 absorbance [27]. It is caused by methoxy groups of 3-GPTMS and 3-APTMS thus it can be concluded that there may be small amount of unreacted 3-GPTMS and 3-APTMS.

Immbilization efficiencies and catalytic activities of the immobilized CALB samples were determined. Free lipase was successfully immobilized onto modified RHA, with immobilization efficiencies of 88 % and 91 % for 3-GPTMS and 3-APTMS modification, respectively. Approximately 80 % of the catalytic activities of both of the immobilized lipases were retained after immobilization process. Additionally, specific activities were close to commercially available immobilized CALB (Novozyme 435®) whose catalytic activity for PCL synthesis is known to be 10 U/mg. Additionally, when compared to free form of CALB, the specific activity values were improved by 1.8 and 1.7 folds for 3-GPTMS and 3-APTMS modification respectively. Thus, immobilized CALB onto 3-GPTMS and 3-APTMS-modified RHA are thought to be promising alternatives to Novozyme 435® for PCL synthesis [14, 55]. The immobilized CALB samples were imaged with SEM. The SEM images revealed a bond-like structure for immobilized CALB onto 3-GPTMS-

modified RHA. This finding is consistent with the information that the immobilization onto an epoxy-activated material is achieved through covalent bonding between the support and the enzyme [14]. On the other hand, such bond structures are not observed for CALB immobilized onto 3-APTMS-modified RHA.

Storage stability of the immobilized enzyme samples were determined in order to find out how long the enzymes can be kept while retaining its catalytic activity. The relative activity changes of the samples were measured periodically for 3 months. For both type of samples, relative activities were dropped to approximatley 45 % after 3 months of storage. Immobilized CALB onto 3-APTMS-modified RHA shoeds a steep decrease after a month of storage while the relative activity of the immobilized CALB onto 3-GPTMS-modified RHA decresed rapidly after 15 days of storage. Additonally, the effects of temperature and pH on the catalytic activity of the immobilized enzyme samples were determined. The optimum temperature of both immobilized enzyme samples onto 3-GPTMS and 3-APTMS-modified RHA is 37 °C. This value is also found to be the optimum value for Novozyme 435® and free lipase. At higher temperatures, activity of immobilized CALB onto 3-GPTMS and 3-APTMS-modified RHA decreases rapidly, dropping around 50 % of the highest value at 37 °C. Both CALB immobilized on 3-GPTMS-modified and 3-APTMS-modifed RHA are thermally stable between 0 – 50 °C, which is a narrower interval with respect to free lipase and Novozyme 435®. Optimum pH of both immobilized CALB samples were pH 7.0. 3-APTMS modification is found to be more efficient than 3-GPTMS modification at basic region. Also, both CALB immobilized onto 3-GPTMS and 3-APTMS-modified RHA have higher activity than free enzyme. Immobilized CALB samples have higher activity than Novozyme 435® at basic regions. At acidic regions Novozyme 435® is more active. It is concluded that these immobilized lipases can be an alternative to Novozyme 435® for catalysis in basic reaction mediums. The pH stability of CALB immobilized onto 3-APTMS-modified RHA is similar to that of Novozyme 435®. They are stable between pH 4.5 – 8.5. On the other hand, immobilized CALB onto 3-GPTMS-modified RHA is found to be stable between pH 4.5 – 7.5. Operational stability of the immobilized enzyme samples were detremined since it is an important that the immobilized enzymes are suitabe for repeated use. 50 % of their initial catalytic activities were retained after 14 cycles of use for both immobilized CALB samples

Apart from temperature and pH, silanization agent concentration and enzyme loading ratio were examined in order to observe the effects on immobilization efficiency and catalytic activity. The immobilization efficiencies are similar to each other; the change of silanization agent concentration and enzyme loading ratio do not have significant effects on the immobilization efficiencies. However, high concentrations of silanization agent or enzyme are not preferred due to economic concerns. Catalytic activities showed varieties unlike immobilization efficiencies. Especially, at high silanization agent concentration catalytic activities of both CALB immobilized onto 3-GPTMS and 3-APTMS-modified RHA decreased approximately 50 %. For immobilized CALB onto 3-GPTMS-modified RHA, 15 % silanization agent concentration and 20 % enzyme loading is decided to be the optimum ratio, by considering both immobilization efficiencies and catalytic activities. The immobilization efficiencies of the CALB samples immobilized onto 3-APTMS-modified RHA are generally higher than the immobilization efficiencies of CALB samples immobilized onto 3-GPTMS-modified RHA. For, immobilized CALB onto 3-APTMS-modified RHA, 10 % silanization agent concentration – 5 % enzyme loading is found to have good immobilization efficiency and catalytic activity than 15 % silanization agent concentration and 20 % enzyme loading.

In conclusion, these newly immobilized enzymes have some promising properties. Firstly, immobilization onto modified RHA is successful for both modifications, yielding high immobilization efficiency. Thus, RHA can be said to be a good choice of support material for enzyme immobilization. In addition, most of the catalytic activity of free lipase was retained for both modifications after immobilization process. The immobilized lipases have temperature and pH stabilities; they are operable at a considerably wide range of pH and temperature. The reusability and shelf life of the immobilized lipases are found to be promising.

However, the findings of this study may be basis for further studies. The properties of the immobilized lipase samples can be determined or improved for specific applications of lipases, for example for polymer synthesis. Such studies are available in the literature investigating immobilized CALB-catalyzed PCL synthesis [52, 54, 64-66]. Furthermore, the findings of this study can be widened by considering few more parameters. For example, immobilization method is an important parameter that affects the immobilized enzyme properties. In this study, immobilization is

achieved by physical adsorption; different methods of immobilization can be applied. Also, as part of parametric studies, immobilization time and stirring rate can be evaluated. One of the factors affecting the properties of immobilized enzyme is the type of silanization agent. The silanization agents used in this study can be replaced with other types of silanization agents.

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APPENDICES

APPENDIX A: Standard Curve

APPENDIX A

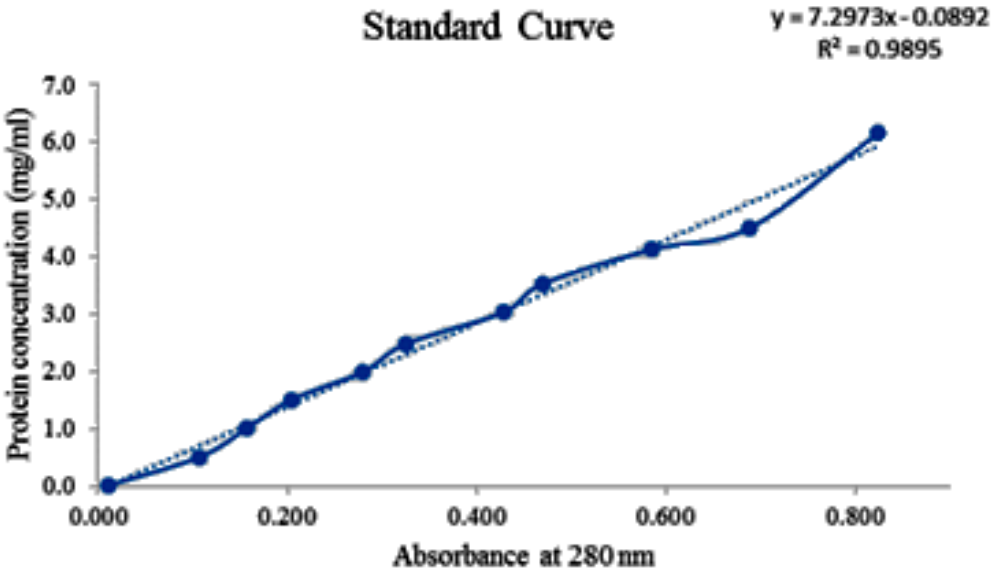

Figure A.1: Standard curve used for UV calculation.

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PUBLICATIONS, PRESENTATIONS AND PATENTS ON THE THESIS:

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